Alpha-Amylase and pullulanase from Thermopallium natronophilum

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Award date:
1998

Awarding institution:
University of Bath

α-AMYLASE AND PULLULANASE FROM *Thermopallium natronophilum.*

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α-Amylase and pullulanase from *Thermopallium natronophilum.*

submitted by Carl R. Thompson
for the degree of PhD
of the University of Bath
1998

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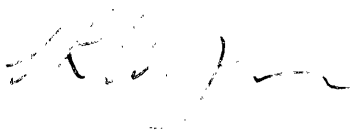

ACKNOWLEDGEMENTS

I would like to thank Prof Michael Danson and Dr David Hough for their guidance and encouragement throughout the project. I would also like to thank Drs Marga Herweijer and Brian Jones for their input and enthusiasm.

I would also like to acknowledge Nicola Ditchburn, for being there, through thick and thin. I would like to thank my Mum and Dad for supporting me to get this far.

And last, but not least, I would like to thank the members of Lab 1.33, past and present, for making my time in Bath so enjoyable.

ABSTRACT

Thermopallium natronophilum produces two enzymes capable of hydrolysing starch; these enzymes have been purified to homogeneity using a combination of gel filtration, anion-exchange and affinity chromatography. The two enzymes have been identified as an α-amylase and a pullulanase type-I, and they specifically hydrolyse α-1,4- and α-1,6-glycosidic linkages, respectively.

The two enzymes have been characterised with respect to their size, sub-unit composition and activity properties. The α-amylase and pullulanase are both monomers and have M_r of 83,000 and 87,000, respectively, according to gel filtration and SDS-PAGE analysis. The α-amylase is a calcium-dependant thermoalkalophilic enzyme, with optimal activity observed at 80 °C and pH 10. The pullulanase has a higher temperature optimum and a more neutral pH optimum, with optimal activity observed at 90 °C and pH 7.5. The α-amylase hydrolyses starch to a number of different size dextrins whereas the pullulanase hydrolyses pullulan to maltotriose. Thermal inactivation studies show that the α-amylase is more thermostable at and above its temperature optimum, compared to the pullulanase.

Limited internal amino acid sequence has been obtained for the two hydrolases. The α-amylase contains the peptide sequence, Ile-Gly-Leu-Pro-Ser-Val-Met-Thr-Glu-Pro-Trp-Asn-Pro-Ile-Gly-Gly-Ser-Asn-Trp-Ile-Phe-Asp-Met-Met-Leu-Ile-Arg and the pullulanase contains the sequence, Tyr-Ile-Gly-Asp-Gly-Ala-Trp-Glu-Ala-Val-Leu-Glu-Gly-Asp-Asp-Glu-Gly-?-Phe-Tyr-Arg. The nucleotide sequence responsible for the α-amylase protein fragment has been amplified and its sequence determined.

This work also outlines the development of a labelling procedure for the production of a highly specific probe using a short nucleotide sequence, using PCR. A Southern blot of restriction digested *T. natronophilum* genomic DNA, revealed a 4.5 kb *Eco* RI cut fragment, which could contain whole or part of the α-amylase gene.

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ABBREVIATIONS

α-cyclodextrin	cyclohexaamylose
ANSA	3-amino,5-nitrosalicylic acid
BCA	bicinchoninic acid
β-cyclodextrin	cycloheptaamylose
bp	base pairs
BSA	bovine serum albumin
CTAB	hexadecyltrimethyl ammonium bromide
Da	daltons
ddNTP	di-deoxy-nucleotide triphosphate
dATP	deoxy-adenosine triphosphate
dCTP	deoxy-cytosine triphosphate
dGTP	deoxy-guanosine triphosphate
DNA	deoxyribonucleic acid
DNP-Lysine	dintrophenyl-lysine
DNSA	3,5-dinitrosalicylic acid
dNTP	deoxy-nucleotide triphosphate
dp	degree of polymerization
dTTP	deoxy-thymidine triphosphate
EDTA	(disodium) ethylenediamine tetraacetate
EGTA	ethylene glycol-bis(β-aminoethyl ether) N, N, N′, N′-tetraacetic acid
FPLC	fast liquid protein chromatography
γ-cyclodextrin	cyclooctaamylose
gDNA	genomic DNA
HEPES	(N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid])
HPLC	high performance liquid chromatography
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase pairs
kDa	kilodaltons
LB	Luria-Bertani
MES	(2-[N-morpholino]ethanesulphonic acid)
MOPS	3-(N-morpholino) propanesulphonic acid
M_r	relative molecular weight
PAGE	polyacrylamide gel electrophoresis
PEG (8000)	polyethyleneglycol (molecular weight)
PCR	polymerase chain reaction
pfu	plaque forming unit

PVDF	polyvinylidene fluoride
RNAse A	ribonuclease A (EC 3.1.27.5)
RP	reverse phase
rRNA	ribosomal ribonucleic acid
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
SSC (20x)	3 M NaCl, 0.3 M sodium citrate (pH 7.0)
TAE	40 mM Tris-acetate, 1 mM EDTA
TE	10 mM Tris-HCl, 1 mM EDTA
TEMED	N, N, N', N" tetramethylethylene diamine
Tris	tris-(hydroxymethyl)-methylamine
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

AMINO ACIDS

One letter code	Three letter code	Amino acid	One letter code	Three letter code	Amino acid
A	Ala	Alanine	M	Met	Methionine
C	Cys	Cysteine	N	Asn	Asparagine
D	Asp	Aspartate	P	Pro	Proline
E	Glu	Glutamate	Q	Gln	Glutamine
F	Phe	Phenylalanine	R	Arg	Arginine
G	Gly	Glycine	S	Ser	Serine
H	His	Histidine	T	Thr	Threonine
I	Ile	Isoleucine	V	Val	Valine
K	Lys	Lysine	W	Trp	Tryptophan
L	Leu	Leucine	Y	Tyr	Tyrosine

To Dad

&

In memory of Mum and Grandad

CHAPTER 1

Introduction

1.1 OVERVIEW

The enzymes under investigation are α-amylase and pullulanase (type I) from *Thermopallium natronophilum*. Firstly the concept of the phylogenetic tree will be described, followed by the placement of *T. natronophilum* within this scheme. The role of α-amylases and pullulanases will then be discussed, followed by a brief insight into the similarities of these enzymes studied from other micro-organisms. Finally, possible industrial applications of carbohydrate hydrolases will be high-lighted.

1.2 THE PHYLOGENETIC TREE

The original concept of life branching into two distinct domains was based purely on phenotypic characteristics; however this scheme has now been redesigned as a result of molecular biological studies. Phylogenetic analysis using genetic sequences has proved to be a more useful and reliable means than more classical indicators such as fossil record, morphology and biochemistry of the organisms. These classical indicators are poor tools for making inferences about evolutionary relationships between the organisms. Once a suitable medium had been chosen, a specific template needed to be studied from which evolutionary relationships between organisms could be inferred. Such templates would have to follow certain criteria, firstly, it must be ubiquitous to all living organisms and secondly, the sequence of interest needs to evolve slowly so that the relatedness of distant species can be determined. Woese and Fox [1977 and 1978] chose Ribosomal RNA (rRNA), in particular 16S and 18S rRNA, to act as such a template. Initial studies revealed that all living organisms clustered in to three primary kingdoms ("urkingdoms"), named the eubacteria, the eukaryotes and the archaebacteria. Further work indicated that the archaebacteria were more related to the eukaryotes than the eubacteria. In 1990 Woese *et al.* proposed that a new taxon should precede the level of kingdom, called "domain", and that life exists in three domains: Bacteria, Archaea and Eukarya, each of which contain at least two kingdoms.

Phylogenetic trees are a schematical representation of evolution and the relatedness between organisms (Figure 1.1). Phylogenetic trees are constructed using sequence alignments that produce data for phylogenetic inference. The most popular phylogenetic

inference method is Parsimony as it is the most basic method and the data produced are easily interpreted. Parsimony literally means stingiest and the theory depicts that simple explanations should prioritise the more complex ones. Therefore, the parsimonious tree displays the minimalist evolutionary changes compared to more sophisticated methods such as Maximum Likelihood. Maximum Likelihood is a more complex statistical method which applies factors such as unequal rates of nucleotide substitution in different lineages, site-specific rate variability and the distribution of these variable sites. Due to the more refined data, this method is gaining popularity amongst the workers in the field.

The phylogenetic tree is rooted in order to highlight the primitiveness and relative branchpoints of the three domains of life. The tree is rooted by the Dayhoff strategy, whereby the amino acid sequences of pairs of paralogous proteins, assumed to have arisen by duplication events and whose common ancestor predates the most recent universal ancestor, are compared. The proteins studied were the α and β subunits of ATPases and the translation elongation factors EF-1α (Tu) and EF-2 (G), each showing high sequence similarity across the three domains of life [Gogarten *et al.* 1989, Iwabe *et al.* 1989]. Thus the root can be positioned where the two genes are thought to have diverged.

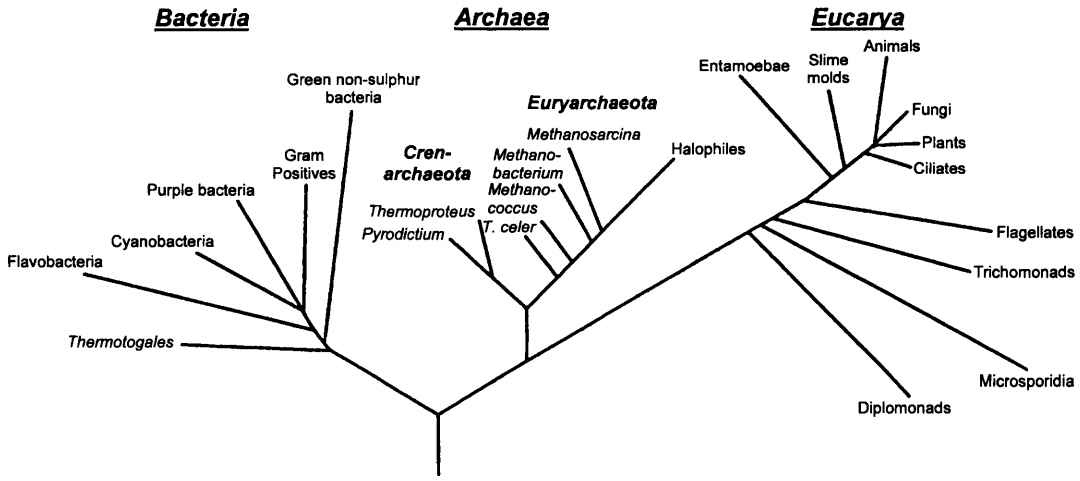


Figure 1.1 The universal phylogenetic tree, as derived by phylogenetic studies of 16s rRNA sequences Reproduced from Wheelis *et al.* [1992] (the tree was rooted as described by Iwabe *et al.* [1989])

1.3 *Thermopallium natronophilum*

Thermopallium natronophilum was isolated in 1992 from soda spring sediment and bottom mud from Lake Bogoria which is located in the Kenyan-Tanzanian Rift Valley. The strains cultured from these sources grow in a pH range from pH 7.2 to pH 10.5 and temperature range from 52 °C to 78 °C. Maximum cell yield is obtained at pH 8.8 to pH 9.5 and 63 °C to 64 °C. *T. natronophilum* cells are rod-shaped and possess an outer sheath-like membrane made of protein, known as a "toga" (Figure 1.2). These initial morphological studies classified the strains as *Thermotogales*, and subsequent 16s rRNA analysis showed that the organism was more closely related to *Fervidobacteria* than *Thermotogales* (Figure 1.3). However, the alkalophilic phenotype and a difference in sequence homology of almost 10 % indicated that a separated genus needed to be erected to accommodate these strains. The assignment '*Thermopallium natronophilum*' (Soda-loving hot coats) has been proposed to include these strains [Duckworth *et al.* 1996].

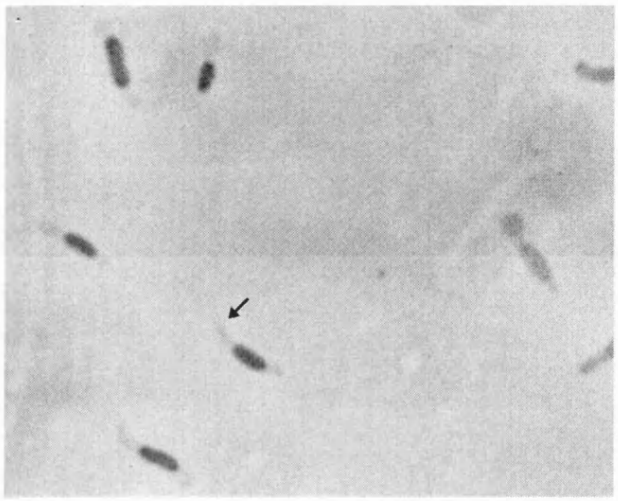
1.4 THE SUBSTRATES

Polymeric substrates such as starch are abundant in nature and provide a valuable and renewable source of carbon as well as energy. A diverse range of fungi, yeast, bacteria and archaea are capable of hydrolysing such complex carbohydrates by producing enzymes with a wide range of specificity.

1.41 Starch

Starch occurs in semi-crystalline form in granules which vary in size and shape depending on the source. Starch is composed of two structures, amylose (15 - 25 %) and amylopectin (75 - 85 %). Amylose is a linear macromolecule consisting of 1,4-linked α-D-glucopyranose residues (Figure 1.4a). The chain length varies from several hundred to 6000 residues. The direction of the chain is determined by the reducing and non-reducing ends. The reducing end is formed by the free C_1 hydroxyl group. Amylopectin, similar to amylose, consists of α-1,4-linked glucose molecules, but in addition contains α-1,6-linkages which form branch-points (Figure 1.4b). These branch-points occur every 17 - 26 glucose molecules; thus the proportion of α-1,6-linkages in amylopectin is approximately 5 %. Amylopectin has a molecular mass of between 10^6 and 10^9 making it one of the largest biological molecules known.

(a)



(b)

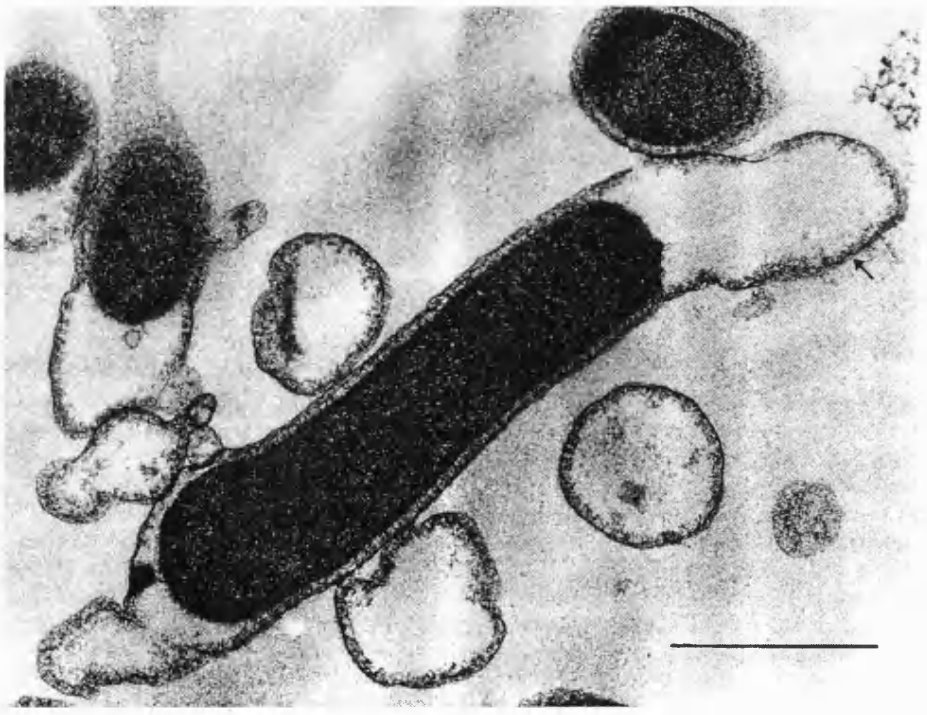


Figure 1.2 Morphology of *T. natronophilum* and related organism

(a) *T. natronophilum* photographed using light microscopy. (b) *Thermotoga maritima* photographed using electron microscopy (Reproduced from Huber *et al.* [1986]). Arrows indicate the toga, which can be seen ballooning at the poles of the rod-shaped cells. Bar, 1μm.

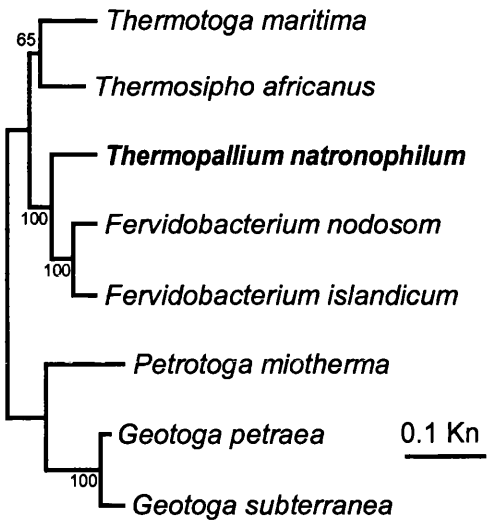


Figure 1.3 Un-rooted phylogenetic tree indicating the relationship between *Thermopallium natronophilum* and representatives of the bacterial order *Thermotogales*
(Reproduced from Duckworth *et al.* [1996])

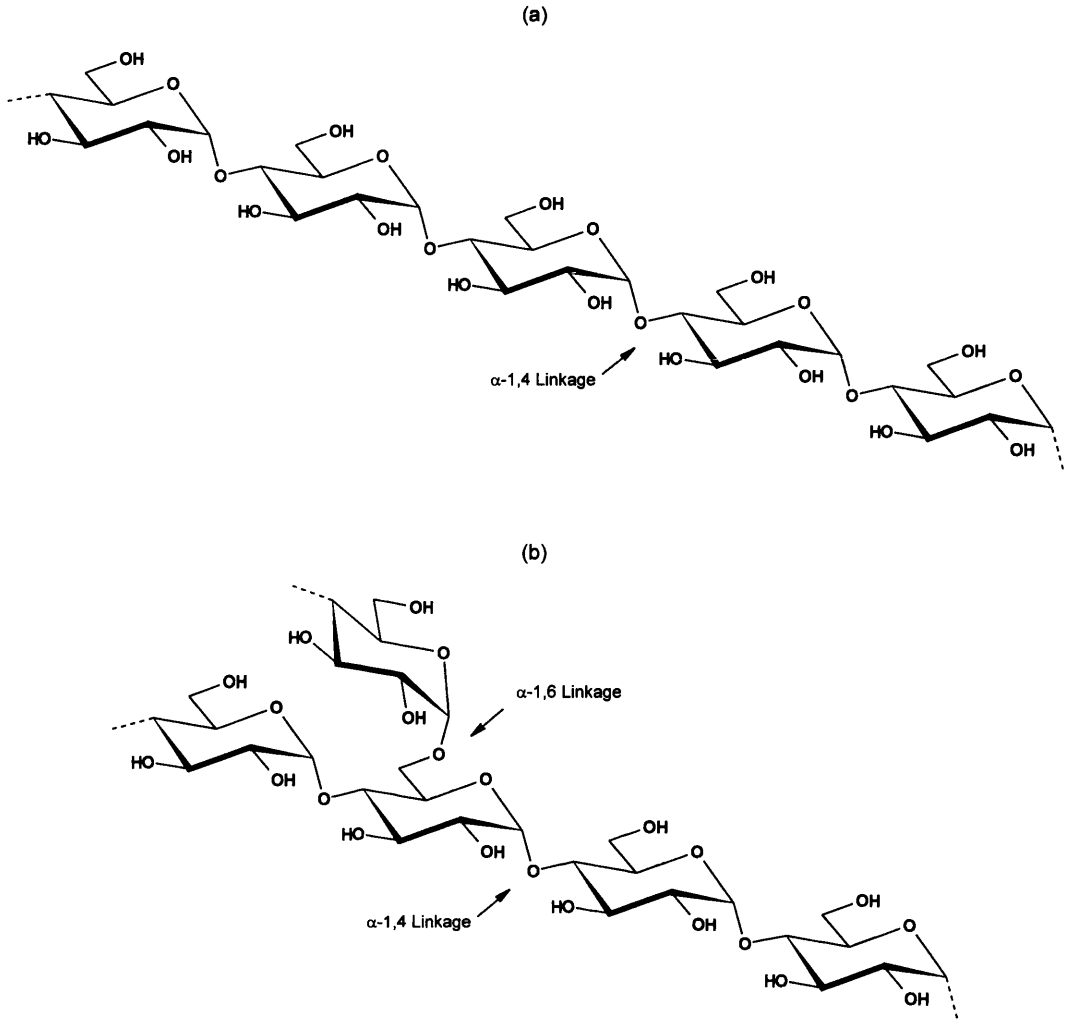


Figure 1.4 The structure of starch

(a) Amylose is composed of glucose molecules interlinked by α-1,4-glycosidic bonds. (b) Amylopectin contains α-1,6-glycosidic bonds in addition to α-1,4-glycosidic bonds.

1.4.2 Pullulan

Pullulan is an α-D-glucan produced by the yeast *Aureobasidium pullulans.* Like amylopectin, pullulan consists of both α-1,4- and α-1,6-linkages (Figure 1.5). The structure of the molecule is very repetitive and consists of approximately 480 maltotriose units linked by α-1,6-D bonds. Because of the high proportion of α-1,6-linkages in pullulan (33.3 %) it is commonly used as a substrate for the study of starch de-branching enzymes.

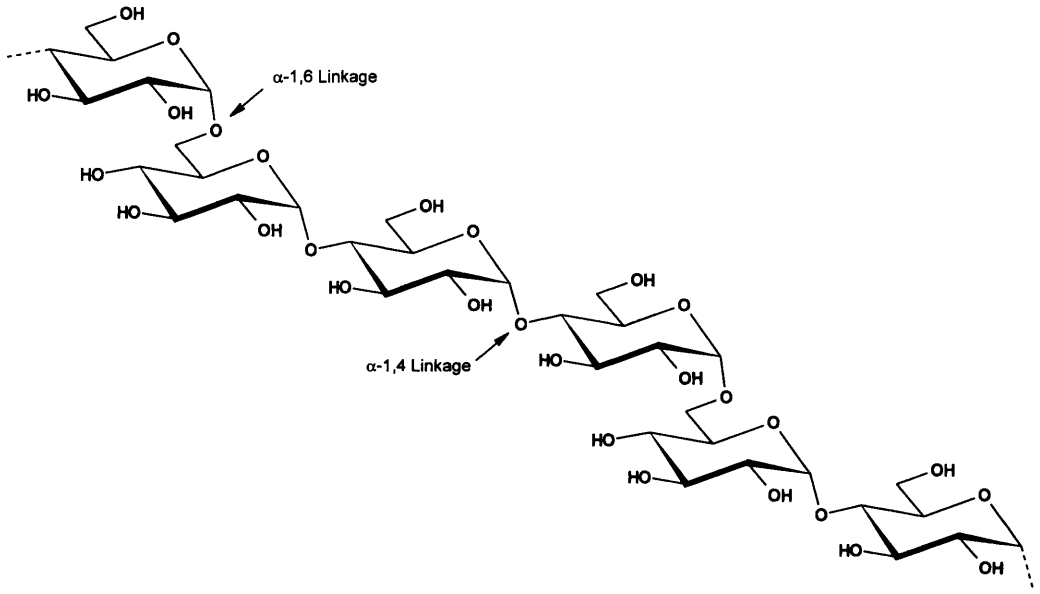


Figure 1.5 The structure of pullulan

Pullulan consist of a repeating maltotriose unit intralinked with α-1,4-glycosidic bonds and interlinked with α-1,6-glycosidic bonds.

1.5 THE STARCH HYDROLASES

1.5.1 Role of starch hydrolases

Starch and pullulan are storage carbohydrates, whereby energy is stored during periods of plentiful energy supply. Organisms possessing enzymes capable of hydrolysing these storage compounds, break the complex carbohydrates to glucose, which is a ubiquitous energy source for all heterotrophic organisms possessing a glycolytic or similar catabolic pathway.

1.5.2 The α-amylase family

Due to the rather complex structure of starch and related polysaccharides, it's not surprising to learn that a number of enzymes are able to metabolise it into smaller, less complex oligosaccharides. The three best known enzymes that facilitate the hydrolysis of starch are α-amylase, β-amylase and γ-amylase (better known as glucoamylase), but despite their similar functions these enzymes are only distantly related [Janecek 1994]. They are structurally different, and the mechanistic action of the αamylase is also different from that of the β- and γ-amylases. In addition to those enzymes which specifically hydrolyse starch there are enzymes that hydrolyse starch related polysaccharides, and these enzymes are also generally classed as starch hydrolases. Based on Hydrophobic Cluster analysis of the amino acid sequences [Gaboriaud *et al.* 1987, Lemesle-Varloot *et al.* 1990], the starch hydrolases have been classified together with the rest of the O-glycosyl hydrolases (E.C. 3.2.1.x), which currently consists of 66 families of enzymes [Bairoch, A. URL: http://expasy.hcuge.ch/cgi-bin/lists?glcosid.txt]. With the exception of the β- and γ-amylases, the starch hydrolases are grouped into the family 13 glycosyl hydrolases. In addition, a few transferases (E.C. 2.4.1.x) are classified together with them due to their glycosyl hydrolase/transferase activity. All these are members of the α-amylase family which currently covers approximately 20 different enzyme specificities (Table 1.1).

EC	Enzyme/Protein
3.2.1.1	α-Amylase
3.2.1.10	Oligo-1,6-glucosidase
3.2.1.60	Maltotetraohydrolase
2.4.1.19	Cyclodextrin glycosyltransferase
3.2.1.20	α-Glucosidase
3.2.1.41	Pullulanase
3.2.1.1/41	Amylopullulanase
3.2.1.54	Cyclomaltodextrinase
3.2.1.68	Isoamylase
3.2.1.70	Dextran glucosidase
3.2.1.93	Trehalose-6-phosphate hydrolase
3.2.1.98	Maltohexaohydrolase
3.2.1.116	Maltotriohydrolase
3.2.1.133	Maltogenic amylase
3.2.1.135	Neopullulanase
	Maltopentaohydrolase
	Maltooligosyltrehalose hydrolase
2.4.1.18	Glucan branching enzyme
2.4.1.25	Amylomaltase
2.4.1.25/3.2.1.33	Glucan debranching enzyme
	Maltooligosyltrehalose synthase
2.4.1.5	Glucosyltransferase
	Amino acid transport-related protein
	4F2 Heavy-chain cell surface antigen

Table 1.1 The members of the α-amylase family

The EC numbers are given if known. The members are ordered in the following manner: the members with cyrstallographically known 3D structure → hydrolases → transferases → glucanotransferase that has been proposed to contain a circularly permuted version of the $(\alpha/\beta)_8$-barrel → proteins without catalytic function. Reproduced from Janecek [1997].

1.5.3 α-Amylase (1,4 α-D-glycan, 4-glucanhydrolase, E.C. 3.2.1.1)

α-Amylases are widely distributed and can be found in plants, animals and micro-organisms. α-Amylase is an endoacting enzyme which produces oligosaccharides and glucose as end-products by hydrolysing α-1,4-glycosidic linkages in a random manner. The enzyme catalyses multi-chain attack as well as multiple attack on the same chain [French 1981]. Complete hydrolysis of amylose yields maltose and glucose. The anomeric carbon atom in all products formed has the α-D configuration. α-Amylases are not able to hydrolyse the α-1,6-linkages found in branched substrates such as amylopectin and glycogen. Despite this, the enzyme is capable of bypassing branch-points. Thus, the action of the α-amylase on branched substrates results in the formation of α-limit dextrins. α-Amylases are also referred to as saccharifying and liquefying enzymes. Saccharifying α-amylases reduce viscosity of starch solutions less than liquefying α-amylases (most bacterial α-amylases) and repetitively attack the substrate. Most α-amylases have a significant requirement for calcium ions, the effect of which increases temperature optima and thermostability in the presence of substrate. α-Amylases are widely distributed among micro-organisms including aerobic and anaerobic Bacteria and Archaea as well as fungi (Table 1.2).

The group of organisms which has invoked the majority of attention are the Gram positive bacteria, *Bacillus*. The *Bacillus* α-amylases are characterised by a wide range of temperature and pH optima. Although many of the *Bacilli* grow at mesophilic temperatures they produce thermotolerant α-amylases which in many cases have temperature optima much higher than the upper temperature limit for growth of the organism. However for the most part, the *Bacillus* amylases possess pH optima that fall somewhere between slightly acidic and neutral. There are exceptions to this rule; for example, *B. acidophilus* as the name suggests thrives at low pH (pH 3.5) and produces an extracellular α-amylase which is optimally active at pH 4.5 and 60 - 75 °C [Boyer *et al.* 1979, Koivula *et al.* 1993, Schwermann *et al.* 1994]. Three other thermoacidophilic starch hydrolases have been isolated from *B. acidocaldarius* strains Agnano 101 [Buonocore *et al.* 1976] and A-2 [Kanno 1986] and from *Bacillus* sp. 11-1S [Uchino 1982]. Starch hydrolases from these organisms exhibit lower pH optima, falling between pH 2 - 3.5 and similar temperature optima of 70 - 75 °C.

There are also thermoalkalophilic α-amylases produced by alkalophilic *Bacilli* strains. The most notable are *Bacillus* sp. GM8901 [Kim *et al.* 1995] and *Bacillus* sp. IMD170 [McTigue *et al.* 1994, McTigue *et al.* 1995]. GM8901 grows at pH 10.5 and 50°C and produces an α-amylases which is most active at 60 °C and pH 11 - 12, which is one of the highest pH optima amongst the starch hydrolases. IMD170 α-amylases has a lower pH and

temperature optima compared with the GM8901 enzyme of pH 10 and 40 °C, respectively. Amylases produced by alkalophilic bacteria does not necessarily have a high pH optimum. For example, Gram positive alkalophilic strain 163-26 grows optimally at pH 9.7, but produces an α-amylase with a pH optimum of 6.6, though admittedly it does retain 60 % of total activity up to pH 10.5.

Amongst the other micro-organisms producing α-amylases is a psychrophilic bacterium, *Alteromonas haloplantis* strain A23, which grows at 4 °C although the α-amylase it produces exhibits a temperature optimum of 25 °C and possesses less than 20 % of its optimal activity at 4 °C [Feller *et al.* 1992]. However, it is postulated that this is compensated for by the elevated catalytic efficiency which is observed at lower temperatures for this enzyme when compared with the α-amylase from pig pancreas [Feller *et al.* 1994]. Other sources of thermophilic α-amylases include archaeal strain TY, archaeal strain TYS, *Desulfurococcus mucosus*, *Fervidobacterium pennavorans*, *Thermococcus celer* [Canganella *et al.* 1994], *Clostridium thermohydrosulfuricum* [Melasniemi 1987], *Clostridium thermosaccharolyticum*, *Thermoanaerobacter finnii*, *Thermoanaerobacter ethanolicus*, *Thermobacteriodes acetoethylicus* [Koch *et al.* 1987] *Noscardia asteroides* [Stevens *et al.* 1994], *Pyrococcus furiosus* [Laderman *et al.* 1993a, Laderman *et al.* 1993b, Koch *et al.* 1990, Dong *et al.* 1997, Jørgensen *et al.* 1997], *Pyrococcus woesei* [Koch *et al.* 1991], *Streptomyces* sp. T01 [Mellouli *et al.* 1996], *Streptomyces thermoviolaceous* strain CUB74 [Bahri and Ward 1993], *Thermoactinomyces* sp. [Obi and Odibo 1984], *Thermococus profundus* strain DT5432, *Thermomyces Lanviginosus* [Jensen and Olsen 1992], *Thermotoga maritima* [Schumann *et al.* 1991] and thermophilic bacterium V2 [Shinomiya *et al.* 1982]. The majority of these α-amylases have activity optima between 50 - 80 °C and pH 5 - 7.5. There are however exceptions to this rule amongst the archaeal organisms. Archaeal strains TY and TYS, *D. mucosus*, *P. furiosus* and *P. woesei* produce α-amylases with temperature optima of 100 °C and even more astonishing in the cases of the *Pyrococcus* enzymes is that they possess remarkable thermal stability and can withstand autoclaving. The *P. furiosus* enzyme has a relatively short half-life of 2 hours at 120 °C and 5 bar [Koch *et al* 1990]. compared with the *P. woesei* enzyme which has a half-life of 5 hours under the same conditions [Koch *et al.* 1991].

Many of the previously mentioned enzymes require calcium ions to be catalytically active or to improve stability and indirectly catalytic activity at higher temperatures. An α-amylase from *Bacillus licheniformis* MY10 [Kumar *et al.* 1990] was shown to be inhibited by the presence of calcium. However the vast majority do require calcium ions.

Organism	Reference(s)
Alteromonas haloplantis	Feller *et al.* [1992], Feller *et al.* [1994]
Archaeal strain TY	Canganella *et al.* [1994]
Archaeal strain TYS	Canganella *et al.* [1994]
Alkalophilic Gram positive bacterium	Candussion *et al.* [1990]
Bacillus acidocaldarius	Schwermann *et al.* [1994], Koivula *et al.* [1993]
Bacillus alcalophilus subsp. *halodurans* ATCC21591	McTigue *et al.* [1994]
Bacillus amyloliquefaciens	Kochnar and Dua [1990]
Bacillus brevis	Kim *et al.* [1992]
Bacillus licheniformis	Takasaki *et al.* [1994]
Bacillus licheniformis strain ATCC6598	Joyet *et al.* [1992]
Bacillus licheniformis strain CUMC305	Krishnan and Chandra [1983]
Bacillus licheniformis strain My10	Kumar *et al.* [1990]
Bacillus licheniformis strain NCIB6346	Morgan and Priest [1980]
Bacillus licheniformis strain RP01	Piggott *et al.* [1984]
Bacillus licheniformis strain TCRDC-B13	Bajpai and Bajpai [1987]
Bacillus sp. IMD170	McTigue *et al.* [1994], McTigue *et al.* [1995]
Bacillus sp. NCIB11203	McTigue *et al.* [1994]
Bacillus sp. strain GM8901 (alkalophilic)	Kim *et al.* [1995]
Bacillus sp. strain JF	Jin *et al.* [1992]
Bacillus Stearothermophilus	Brosnan *et al.* [1992]
Clostridium thermohydrosulfuricum	Melasniemi [1987]
Clostridium thermosaccharolyticum	Koch *et al.* [1987]
Desulfurococcus mucosus	Canganella *et al.* [1994]
Fevidobacterium pennavorans	Canganella *et al.* [1994]
Noscardia asteroides	Stevens *et al.* [1994]
Pyrococcus furiosus	Laderman *et al.* [1993a], Laderman *et al.* [1993b], Koch *et al.* [1990], Dong *et al.* [1997], Jorgensen *et al.* [1997]
Pyrococcus woesei	Koch *et al.* [1991]
Streptomyces sp. T01	Mellouli *et al.* [1996]
Streptomyces thermovilolaceous strain CUB74	Bahri and Ward [1993]
Thermoactinomyces sp.	Obi and Odibo [1984]
Thermoanaerobacter ethanolicus	Koch *et al.* [1987]
Thermoanaerobacter finnii	Koch *et al.* [1987]
Thermobacteriodes acetoethylicus	Koch *et al.* [1987]
Thermococcus celer	Canganella *et al.* [1994]
Thermococcus profundus strain DT5432	Jensen and Olsen [1992]
Thermomyces lanviginosus	Jensen and Olsen [1992]
Thermotoga maritima	Schumann *et al.* [1991]
Thermophilic bacterium V2	Shinomiya *et al.* [1982]

Table 1.2 Organisms that produce α-amylase

1.5.4 Pullulanase type I (α-dextrin 6-glucanohydrolase, E.C. 3.2.1.41)

Pullulanase type I hydrolyses α-1,6,-linkages in amylopectin, pullulan and limit dextrins with high specificity. Pullulan is completely hydrolysed in a random fashion to maltotriose, whereas native glycogen is not attacked by the enzyme.

Since the discovery of pullulanase (type I) from *Klebsiella aerogenes* (formerly *Aerobacter aerogenes*) [Bender and Wallenfels 1961] a number of pullulanases have been reported from psychrophilic, mesophilic, thermophilic and alkalophilic organisms (Table 1.3).

An extracellular pullulanase activity has been discovered in an alkalopsychrophilic bacterium, *Micrococcus* sp. 207 [Kimura and Horikoshi 1989]. The enzyme has a remarkably high temperature optimum of 60 °C, which is significant considering that optimum cell growth is at 0 °C. The optimum for pullulanase production is at 17 - 20 °C, at which temperature the pullulanase is still reported to retain activity.

The number of mesophilic bacteria studied for pullulanases seem to be low, perhaps due to the interest in finding thermophilic enzymes suitable for industrial purposes. Like the psychrophilic pullulanase, pullulanases purified from mesophilic bacteria also appear to be thermotolerant, though this is accompanied by low activity when compared to thermostable enzymes from thermophilic bacteria. Such mesophilic bacteria producing thermotolerant pullulanase are *Bacillus acidopullulyticus* [Jensen and Norman 1984], *Bacillus cereus* [Bakshi *et al.* 1992], alkalophilic *Bacillus* No. 202-1 [Nakamura *et al.* 1975] and *Klebsiella aerogenes* [Mercier *et al.* 1972]. These organisms although grown under mesophilic conditions produce thermotolerant pullulanase stable at 50 °C, 55 °C, 60 °C and 70 °C, with respect to *K. aerogenes*, *B.* No. 202-1, *B. acidopullulyticus* and *B. cereus*. Optimal pullulanase activity was observed at pH 4.5 - 5.0, 5.5, 8.5 - 9.0 and 7.0, with respect to *B. acidopullulyticus*, *K. aerogenes*, *B.* No. 202-1 and *B. cereus*.

Pullulanase is also produced by a number of moderately thermophilic micro-organisms, such as *Clostridium thermohydrosulficum* (intracellular and extracellular) [Saha *et al.* 1988, Melasniemi 1987], *Bacillus stearothermophilus* [Kuriki *et al.* 1990], *Thermoanaerobacter finnii*, *Thermobacteriodes acetoethylicus*, *Clostridium thermosaccharolyticum* and *Thermoanaerobacter ethanolicus* [Koch *et al.* 1987], alkalophilic *Bacillus* sp. S-1 and *Micrococcus* sp. Y-1 [Kim *et al.* 1993]. Although these micro-organisms themselves show optimal growth at moderate temperatures (50 - 60 °C), in most cases the pullulanases they produce, of which most are extracellular, exhibit reasonably high temperature optima. Indeed, with the exception of the alkalophilic *Bacillus* sp. and *Micrococcus* sp., the temperature optima are in the range of 75 - 90 °C. Those pullulanases with higher temperature optima also exhibited pH optima close to that of the surrounding media. This

was also the case for the alkalophilic micro-organisms, though their pullulanases had lower temperature optima.

Among the thermophilic micro-organisms that produce pullulanase are *Thermus caldophilus* GK-24 [Kim *et al.* 1996], *Thermus* sp. AMD-33 [Nakamura *et al.* 1989], *Fervidobacterium pennavorans* Ven5 [Koch *et al.* 1997], *Thermococcus celer*, *Desulfurococcus mucosus*, Archaeal strain TYS, Archaeal strain TY, *Fervidobacterium pennavorans* [Canganella *et al.* 1994] and *Pyrococcus woesei* [Rüdiger *et al.* 1995]. The temperature optima of the pullulanases isolated from these thermophiles are naturally the highest of all the pullulanases isolated so far. The optima fall within the temperature range 70 - 100 °C, *D. mucosus*, Archaeal strain TYS, Archaeal strain TY and *P. woesei* all possessing a temperature optimum of 100 °C. The pH optima of these pullulanases from the thermophiles are very similar to those pullulanases isolated from less temperature tolerant micro-organisms and prefer a slightly acidic environment (pH 5.0 - 6.5).

Organism	**Reference(s)**
Archaeal strain TY	Canganella *et al.* [1994]
Archaeal strain TYS	Canganella *et al.* [1994]
Klebsiella aerogenes	Bender and Wallenfels [1961], Mercier *et al.* [1972]
Bacillus acidopullulyticus	Jensen and Norman [1984]
Bacillus cereus	Bakshi *et al.* [1992]
Bacillus stearothermophilus	Kuriki *et al.* [1990]
alkalophilic *Bacillus* No. 202-1	Nakamura *et al.* [1975]
alkalophilic *Bacillus* sp. S-1	Kim *et al.* [1993]
Clostridium thermohydrosulficum	Saha *et al.* [1988], Melasniemi [1987]
Clostridium thermosaccharolyticum	Koch *et al.* [1987]
Desulfurococcus mucosus	Canganella *et al.* [1994]
Fervidobacterium pennavorans	Canganella *et al.* [1994]
Fervidobacterium pennavorans Ven5	Koch *et al.* [1997]
Micrococcus sp. 207	Kimura and Horokoshi [1989]
Micrococcus sp. Y-1	Kim *et al.* [1993]
Pyrococcus woesei	Rüdiger *et al.* [1995]
Thermoanaerobacter ethanolicus	Koch *et al.* [1987]
Thermoanaerobacter finnii	Koch *et al.* [1987]
Thermobacteriodes acetoethylicus	Koch *et al.* [1987]
Thermococcus celer	Canganella *et al.* [1994]
Thermus caldophilus	Kim *et al.* [1996]
Thermus sp. AMD-33	Nakamura *et al.* [1989]

Table 1.3 Organisms producing pullulanase type-I

1.5.5 Enzyme structure

Although there is little sequence identity of the α-amylases between species, sharing approximately 10 % or less identity [Raimboud *et al.* 1989], α-amylases along with pullulanases possess several regions of similarity, some of which contain fairly well conserved residues between species if not the α-amylase family. Those regions of similarity, when compared across the α-amylase family that do not appear to contain conserved amino acids, are mostly replaced by semi-conservative substitutions [Janecek 1992]. This can be seen in the current table of the conserved regions of the members of the α-amylase family (Table 1.4)

Although some of the starch hydrolases lack high identity, the structures that the primary structure form are very similar for the enzymes. The three-dimensional structure comprises eight consecutive parallel β-strands surrounded by eight α-helices, better known as the $(\alpha/\beta)_8$-barrel structure [Brändén 1991] (Figure 1.6a). The barrel domains found in the α-amylase family are however different to those found in Triosephosphate IsoMerase (TIM), which was the first and most "regular" example of an eight-folded parallel $(\alpha/\beta)_8$-barrel motif. The most significant difference is the presence of a small domain, referred to as Domain B, which forms between strand β3 and helix α3 [Klein and Shultz 1991, Qian *et al.* 1993]. This feature is common to each enzyme from the α-amylase family [Janecek 1994]. The $(\alpha/\beta)_8$-barrel portion of the enzyme is referred to as Domain A. As indicated in Table 1.4, the β strands of the α-amylase family type $(\alpha/\beta)_8$-barrel enzymes show the greatest degree of conservation compared to the α-helices. These α helices are relatively variable in length and it has been postulated that this accounts for the various enzyme specificities in the α-amylase family. The β strands are thought to act like a scaffold for residues essential for substrate binding and catalysis [Jespersen *et al.* 1993]. The catalytic residues are found near the C-termini of the barrel β strands and for those structures of the α-amylase family solved they are superimposable [Janecek 1997]. In *Aspergillus oryzae* the two catalytic amino acids are Asp206 and Glu230 in addition, there is an Asp297 which is believed to form a water-mediated hydrogen bond with Glu230 and plays a role in maintaining an elevated pK_a for the residue for the glycosylation step of the reaction [Brzozowski and Davies 1997] (Figure 1.6b). Other α-amylase family $(\alpha/\beta)_8$-barrel enzymes of course have these residues which are well conserved across the spectrum of specificities (Table 1.4). A proposed sequence of chemical states and events have been proposed for the probable catalytic mechanism of glycolsyl hydrolases (Figure 1.7).

The reaction proceeds to the covalent glycosyl-enzyme intermediate by an acid-catalysed addition-elimination reaction, whereby the deprotonated carboxylic residue (Asp206) attacks C_1 of the glycosyl residue displacing the leaving group OR^-, which itself attacks the acidic

residue (Glu230) and leaves as HOR. The presence of the basic, deprotonated carboxylic group (Glu230) promotes ester hydrolysis through an addition-elimination mechanism. The basic residue converts the poor nucleophile, water, into a negatively charged and more nucleophilic hydroxide ion. Unlike acid-catalysed hydrolysis, the base-mediated process (saponification) is driven essentially to completion in free solution. However, with the reactive groups in such close proximity in an enzyme-mediated ester hydrolysis, the reaction proceeds as an equilibrium reaction until the products leave the active site.

As well as catalytic residues, there are a number of virtually invariantly conserved residues which are involved in substrate recognition and binding. As mentioned earlier α-amylases and indeed other α-amylase family enzymes require calcium ions for stability. Two binding sites were established in the *Aspergillus niger* α-amylase. A primary site, which is located at the interface between domains A and B, is involved in maintaining the structural integrity of the active site. The secondary calcium ion binding site is located at the bottom of the substrate binding cleft between domains A and B and involves the catalytic residues themselves. This may explain why there is an inhibitory effect at high calcium concentrations [Boel *et al.* 1990]. The primary site has been well characterised in a number of sources, which include α-amylases from *Bacillus licheniformis* [Machius *et al.* 1995], barley [Kadziola *et al.* 1994] and pig pancreas [Qian *et al.* 1994] and cyclodextrin transferase from *Bacillus circulans* [Klein and schultz 1991]. In the *A. oryzae* α-amylase there are four residues involved in calcium ion binding, three of them are fairly well conserved amongst the α-amylase family, Asn121, Asp175 and His210 (Table 1.2). In addition to calcium ion binding, some α-amylases have been shown to bind chloride ions, such as pig pancreatic [Qian *et al.* 1994], *Bacillus licheniformis* [Machius *et al.* 1995] and *Alteromonas haloplantis* [Feller *et al.* 1992] although many α-amylases appear to be chloride independent [Vihinen and Mäntsälä 1989]. However, Machius and coworkers [1995] have emphasised that the chloride binding regions of other enzymes from the α-amylase family are clear and chloride ions may be present in those structures, although the authors did not report it.

On a macro-molecular scale, most α-amylases and pullulanases isolated to date are composed of a single subunit and have an M_r in the region of 25 - 150 kDa, the majority of which fall between 60 - 90 kDa.

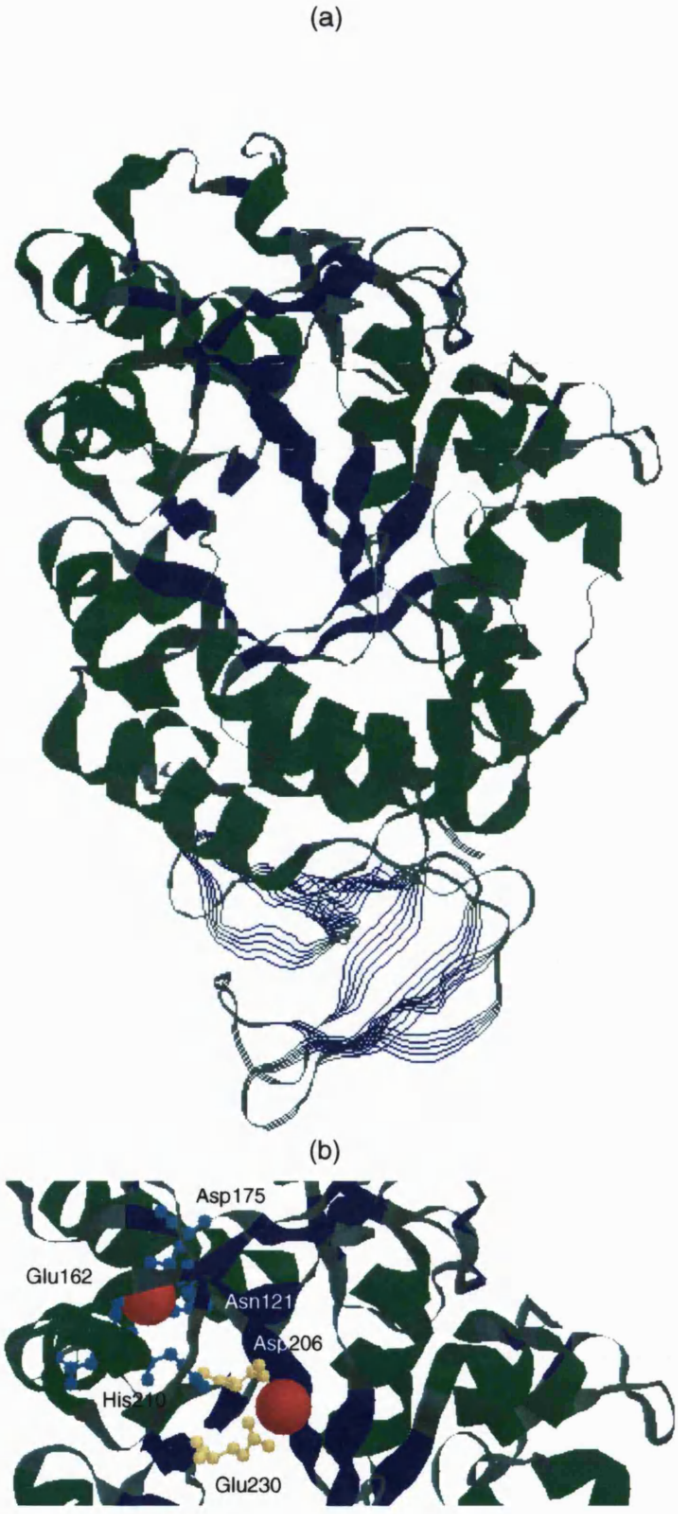


Figure 1.6 3D Crystal structure of *Aspergillus oryzae* α-amylase

(a) The full view showing the structure with the two domains, A and B, α-helices are coloured green, β sheets are coloured blue and connecting loops are coloured blue/green. Domain A is displayed in ribbon form whilst domain B is displayed in strand form. (b) Close-up of the active site, including the two catalytic residues (Asp206 and Glu230) coloured yellow, the calcium binding residues (Asn121, Asp175, His210 and Glu162) coloured cyan and the calcium atoms coloured red. Figures generated using Rasmol (Version 2.5, Molecular Visualisation Program, Glaxo research and development, Greenford, Middlesex, U.K.) and protein database file 6taa.pdb.

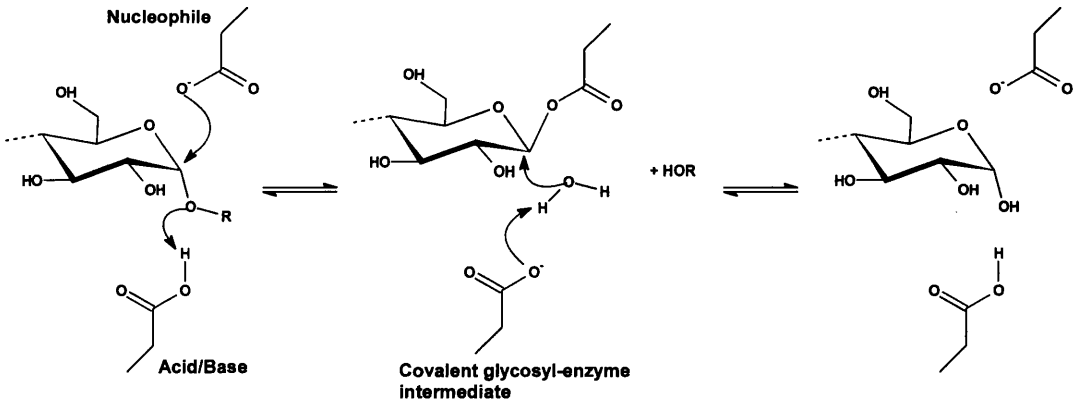


Figure 1.7 Schematical representation of the catalytic mechanism of glycosyl hydrolases (Reproduced and adapted from Brzozowski and Davies [1997])

1.5.5 Assay principle

The assay is based on the procedure outlined by Bernfeld [1955] and is suitable for measuring the activity of amylases and pullulanases and other activities where hydrolysis yields free reducing C1 termini of mono-, oligo- or poly-saccharides. The assay utilises the increasing number of C1 termini following the hydrolysis of starch and related polysaccharides by members of the α-amylase family. The assay is discontinuous and requires the use of a coloured reagent, 3,5-dinitrosalicylic acid (DNSA) which changes colour upon reduction and which can be measured spectrophotometrically at 550 nm. The reaction which takes place is a reduction-oxidation reaction, where the carbohydrate is oxidised and DNSA is reduced. The reaction as well as requiring 4 protons needs heat (100 °C) and high pH for the colour change to occur (Figure 1.8). The C1 terminal glucose, like individual glucose molecules, exists in two forms which are in equilibria, cyclic and acyclic, and it is in the acyclic form in which the reducing group, aldehyde, is responsible for the term "reducing end" when referring to polysaccharides. As the aldehyde is reduced, the equilibrium shifts to the acyclic form, so that eventually all of the free C1 termini are converted. This is accompanied by the reduction of DNSA to 3-amino,5-nitrosalicylic acid (ANSA), and the increase in absorbance at 550 nm. The reaction shown in figure 1.8 shows that one mole of DNSA reacts with one mole of reducing sugar; however, this is not always the case. Some sugars also produce side reactions as well as the one outlined and care must be taken when assaying hydrolytic activity using different sugars [Wang, N. S. URL: http://www.glue.umd.edu/~nsw/ench485/lab4a.htm].

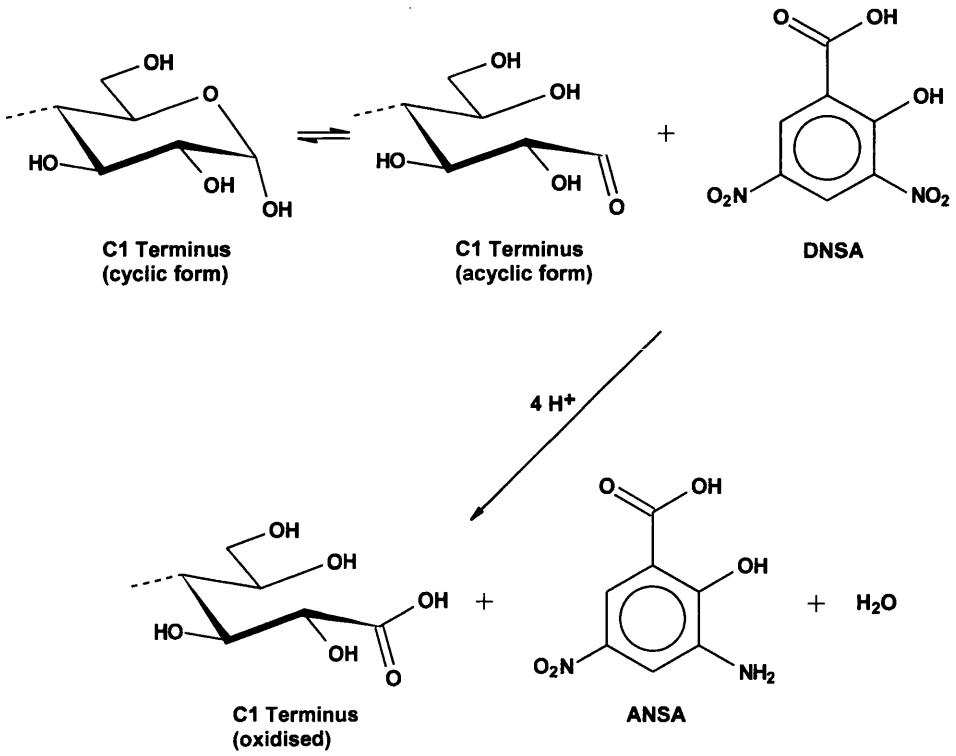


Figure 1.8 Reduction-oxidation reaction involving reducing sugar and DNSA

The C1 terminus (reducing end) is oxidised from an aldehyde to a carboxylic group, whilst DNSA is reduced to ANSA.

1.6 BIOTECHNOLOGICAL APPLICATIONS

1.6.1 Detergent industry

Alkalophilic micro-organisms have already made a notable impact in the application of biotechnology for the manufacture of mass market consumer products. "Biological detergents" are one of the few products of the biotechnology industry used by the ordinary consumer on a day-to-day basis. Alkalophilic and alkalitolerant bacteria are the major contributors of these enzymes found in detergents. Indeed, in 1983 about a quarter of the total world enzyme production was destined for the laundry detergents market. The world-wide market was estimated to be worth $200 million in 1987 and is still growing strongly [Christensen *et al.* 1987]. More recent estimations of the world-wide market for laundry detergents (1996) gives a figure just under $500 million, accounting for one third of the total market for industrial enzymes (URL: http://www.dyadic-group.com/enzymes.htm).

The use of enzymes in laundry processing is not a new idea. In 1913 a German scientist, Otto Röhm was granted a patent for the application of pancreatic trypsin as a laundry aid. In the 1960's a pre-wash laundry detergent, Biotex® which contained an alkaline protease called Alcalase® (Subtilis Carlsberg) produced from *Bacillus licheniformis* was introduced to the market. Although biological detergents gained popularity in both Europe and the U.S., subsequent consumer reaction and unfavourable publicity in conjunction with poor cost effectiveness and performance led to their withdrawal. In the 1980's there was a revival in biological detergents due partly to environmental concerns and energy efficiency, but mainly due to the remarkable improvement in the technology of the products concerned.

The most commonly used enzymes in detergents are proteases and amylases; however, some detergents are now being produced which also contain lipases and cellulases. The function of proteases is to hydrolyses proteins, thus removing proteinaceous stains such as egg and blood etc. However proteinases also have a second function to perform, that is to ensure proteins including those in the detergent are not redeposited onto the fabric. Redeposition of protein onto white fabrics will cause an unclean, greyish decolouration. To increase washing performance at lower temperatures additional enzymes need to be incorporated. Amylases are added to assist proteinases in dissolving protein-starch food stains as well as dispersing starch-based stains. At lower temperatures hydrophobic fatty stains such as those found in cosmetics and oil-based foods are more difficult to remove. Lipases are incorporated into detergents to break-down the hydrophobic fats into smaller hydrophilic fatty acid salts that are easier to remove. Cellulases are also finding their way into laundry detergents. Cellulases exhibit fabric softening and colour brightening properties besides removing soil. Cellulases are believed to achieve these effects by removing or opening up the microfibrils that appear on the surface of cotton fabrics that are due to wear

and washing. Cellulases also have an "anti-piling" effect, whereby loose fibres are cleaved at their branchpoints, preventing them from tangling with others to form "pils". Cellulases do however have a detrimental effect on the fabric and can cause weakening of the fibres due to cotton fibre degradation.

1.6.2 Food industry

Starch processing is a well established business in the U.S. and is concerned with the production of High Fructose Corn Syrup (HFCS) and glucose, which are used as sweeteners. As well as for the food industry, glucose is used to produce ethanol fermentatively which is used as a fuel extender.

The conversion of starch to sugars has undergone impressive development since its modest beginning in the laboratory of a Russian chemist over 175 years ago. The major technological breakthrough occurred in 1946, when Dale and Langois (1940) patented the use of commercially available enzymes and hydrolysed starch in the manufacture of commercial corn syrups from corn starch. The second major technological breakthrough came when the production of HFCS became the first large-scale use of an immobilised enzyme system in the World, which resulted in a significant reduction in production costs [Venkatasubramanian 1978]

Technology over the years has allowed for a totally enzymatic process to be used for the production of these compounds. The first step is concerned with the dextrinisation of starch by liquefaction which takes place at 95 - 105 °C and pH 6.5. *B. licheniformis* α-amylase is used in this step and is required to work at 105 °C for 5 - 10 minutes and 95 °C for 1 - 2 hours. The second step, saccharification, involves the use of glucoamylase which operates at 60 °C and pH 4.5, lasting many hours due to the presence of α-limit dextrins. The third and final step involves the use of glucose isomerase which operates at 60 - 65 °C, pH 7.5 to produce an enriched fructose fraction.

There are improvements that could be made to this process. Firstly, the *B. licheniformis* α-amylase is not ideally thermostable, especially at 105 °C. As it is not possible to reduce the temperature of the liquefaction step, due to the insolubility of starch at lower temperatures, the application of a more thermostable α-amylase would improve the process. Secondly, it is counter cost effective to reduce the temperature in the reactor for the saccharification step, and the incorporation of a more thermostable glucoamylase could prevent this. Finally, the incorporation of a pullulanase in the saccharification step has been shown to speed up the process of α-limit dextrins to glucose and the amount of glucoamylase can be reduced. The reduction of glucoamylase would also reduce the

amount of D-glucose lost as isomaltose and saccharification can be carried out at higher substrate concentrations [Nigam and Singh 1984]. Thirdly, the final step requires the pH to be raised to pH 7.5; this involves the use of ion exchangers to remove large amounts of salts, which is time consuming and costly. Thus, the introduction of an acid-stable glucose isomerase would be beneficial for this process.

1.6.3 Beverage industry

Pectinases, amylases, cellulases and hemicellulases of fungal origin are used in the production of fruit juices and clarification of beers and wines. Thermotolerant, psychrotolerant and acid-tolerant enzymes could perhaps improve these processes. However, the stringent guidelines involving the use of enzymes in food processing are a major obstacle to overcome; this, coupled with a relatively small market, hinders the introduction of new enzymes.

1.6.4 Textile industry

Cotton fibres are strengthened with a starch-based 'size' before weaving, and after the fabric has been woven the starch is removed enzymatically before bleaching and dying, etc. An alkali-tolerant, thermostable amylase could be of use in this application, although with the shift of the textile industry to South-east Asia and the replacement of starch-based 'size' with polyvinylacetate, it would not make economic sense to develop a new enzyme for the process.

1.6.5 Bakery industry

Enzymes are used in the baking industry as bread-improvers, that is not only to improve the texture of the bread but also to extend the shelf-life of the product. This is achieved by the addition of amylases and pullulanases which alter the ability of starch to recrystallise after baking. This helps to retain moisture allowing the loaf to remain fresh for longer. α-Amylase works well but produces a bread crumb with an unacceptable sticky texture. For the enzymes to be effective they need to be active at 70 °C, pH 5 - 5.5. The enzymes also need to be thermostable and active during the baking process for approximately 15 minutes when the temperature inside the bread rises to above 100 °C.

1.6.6 Limiting factors concerning product development

It is perhaps worth a mention that physical characteristics of the enzyme required for industrial purposes are not the only information taken into consideration when developing a new product. There are many other considerations to take into account. Although the enzyme may seem suitable on a small laboratory scale, it may not perform as well as expected in a large-scale reactor system. As well as the importance of performance of the enzyme there is the actual production of the enzyme itself (usually recombinant). Enzyme production must be high and purification quick and cheap (extracellular protein) for the enzyme to be cost effective. Possibly the major factor controlling the cost of producing the enzyme is the consumer. For example, most biological washing powders contain two or more enzymes in large quantities, and the price of these powders have to compare well with non-biological powders for the consumer to buy them.

As well as performance and production there is another hurdle to overcome before the product can be marketed. Like drugs, enzymes, especially those used in the preparation of food stuffs, must undergo expensive strict toxicology studies and be proven to be safe to be approved by the Food and Drug Administration (FDA). This process takes years to complete from the first enzyme characterisation to full scale production and each enzyme can cost up to £10 million to develop. This, coupled with a continually changing market, means that very few enzymes are developed, and the more revolutionary and novel the process *i.e.* the greater the possible market, the easier it is to make the decision to develop. As mistakes are costly, many processes which already have biotechnology incorporated have little incentive to invest in costly development of new products.

1.7 Project aims

1.7.1 Original application

Amylases and xylanases have been isolated and characterised from a number of different organisms. These enzymes, as well as playing an important role within or around the cell, also have industrial applications.

Amylases are used in the food industry (production of high fructose corn syrup and dextrose), the detergent industry (breakdown of starch) and in the textile industry (desizing starch). Xylanases on the other hand are thought to be of use to the paper pulp industry (breakdown of lignin), but as yet the use of the enzyme is not commercially viable as the enzyme is expensive to produce and is inactivated by the extreme conditions in which it is required to work. In all these industrial applications (with the exception of the food industry) two common extremities are experienced, namely high temperature and alkaline conditions; thus, the production of amylases and xylanases that can tolerate such conditions would be ideal.

Recently a bacterium *Thermopallium natronophilum* strain Tg9A has been isolated from a soda spring in Lake Bogoria (pH 8.5 - 10). This new strain is a thermophilic, alkalophilic obligate anaerobe; therefore, this organism would seem to be a candidate for the isolation of such amylases and xylanases for the above industrial applications.

The early stages of the project were to concentrate on isolating and purifying the enzymes, followed by characterisation of the enzymes kinetically (*e.g.* optimum pH and optimum temperature for enzyme activity). Following the purification of the enzymes partial amino acid sequence of the enzymes would be determined to enable primers to be constructed so that the genes could be isolated. It was envisaged that once this work had been carried out Gist Brocades (the CASE collaborating company, based in Delft, The Netherlands) would issue patents for the enzymes. Until the patents had been approved it was unclear what path further work would take.

1.7.2 Supplementary to original application

After initial studies it was clear that studying both amylases and xylanases from *Thermopallium natronophilum*, was an unrealistic goal and a decision was taken to continue studies on the amylases and return to the xylanases only if there was sufficient time.

CHAPTER 2

Materials & Methods

2.1 PROTEIN MATERIALS AND METHODS

2.1.1 Enzymes, reagents and other materials

Acetic acid, butanol, calcium chloride, EDTA, ethanol, glucose, hydrochloric acid, methanol, phosphoric acid, sodium chloride and sodium hydroxide were of the highest quality and were obtained from Fisons, Loughborough, Leicestershire, UK.

Acrylamide/Bis-acrylamide mixture, Ultrapure protogel™, was supplied by National diagnostics, Atlanta GA, USA. Ammonium persulphate and protein molecular weight standards were supplied by Bioraid, Hercules CA, USA. BCA protein estimation reagents were supplied from Pierce, Rockfford IL, USA. Tris was supplied by Gibco BRL, Grand Island NY, USA. Zulkowsky starch was supplied by BDH/Merck, Poole, Dorset, UK.

All other substrates and reagents used in protein methods throughout this work were purchased from Sigma, St. Louis MO, USA.

2.1.2 Growth of *Thermopallium natronophilum*

Growth of the microorganism took place at CAMR (Centre for Applied Microbiology Research) at Porten Down, Salisbury, under the supervision of Prof Richard Sharp and Dr Neil Raven.

The medium used to culture *T. natronophilum* was composed of (per 1 L): 100 ml Castenholz solution A, 10 ml Castenholz solution B, Castenholz solution C, 5 ml Vitamin solution (as per Raven *et al*, [1992]), 1 ml Resazurin solution (1 gL^{-1}, Sigma, St. Louis MO, USA), 2 g Tryptone, 1 g Yeast extract (Difco Laboratories, Detroit MI, USA), 2.5 g Starch, soluble (BDH/Merck, Poole, Dorset, UK), 2 g NaCl, 5 g Sodium bicarbonate and 0.5 g Sodium sulphide $x.H_2O$ (BDH/Merck). The pH was adjusted to pH 8.5 using 1 M HCl.

The media was prepared in the following manner. Soluble starch was autoclaved (121 °C for 15 min) in a glass reservoir bottle as a 5x concentrate solution (4 L of a 12.5 gL^{-1} suspension in water). The bottle was fitted with a gas distribution tube, gas inlet and outlet

filters, an inoculation port and a Sartobran PH filter (Sartorius, Göttingen, Germany) prior to autoclaving. The remaining medium was made up as a 1.25x concentrate (in 16 L of water) with the sodium sulphide being added immediately before adjustment of the pH and filter sterilisation directly into the glass reservoir bottle via the Sartobran PH filter.

The culture vessel was placed in a fan-assisted incubator (Gallenkamp/Fisons, Loughborough, Leicestershire, UK) set at 65 °C and while heating was sparged with oxygen-free nitrogen for 1 h. Anaerobicity was assumed from the decolourisation of the resazurin. The culture was then inoculated with 2 x 100 ml seed cultures prepared using the same medium but grown in closed 160 ml serum bottles.

Cultures were grown in 20 L volumes in glass reservoir bottles without pH control, at 65 °C, under continuous sparging with oxygen-free nitrogen (Distillers MG, Reigate, Surrey, UK) at 0.1 vvm through a P160 gas distribution tube (maximum porosity 160 μm). A 1 % inoculum was used and cultures were terminated when the optical density plateaued (OD_{600} 0.7 - 0.8 and 18 - 21 h growth). No change in pH was observed during growth. Cells were harvested by serial centrifugation (7,277 x *g* for 20 min at 4 °C) in 1 L pots using a H6000-A rotor installed in to a Sorvall RC-3B centrifuge. Cell paste was dispensed into sterile 50 ml conical tubes and stored frozen at -20 °C until transport on solid CO_2 to the University of Bath.

Several cultures have been prepared to date. Yields were generally in the region of 80 g wet weight of biomass from the 20 L batches, equivalent to 4 gL^{-1}.

2.1.3 Protein Estimation

2.1.3.1 Bradford assay

For protein concentrations determined by the method outlined by Bradford [1976], 100 μl of protein sample was incubated with 1 ml of Bradford reagent (0.01 % (w/v) Coomassie brilliant blue G-250, 4.8 % (v/v) ethanol, 8.5 % (v/v) phosphoric acid) at room temperature for 10 min. Protein concentration was determined by comparing sample absorbances to a standard curve constructed using standards of known concentration. Standards contained BSA at concentrations from 0 - 200 μg. The standard curve and sample protein concentrations were calculated automatically by a program installed in the spectrophotometer (LambdaBio, Perkin-Elmer, Norwalk CT, USA).

2.1.3.2 BCA assay

A 100 µl sample was mixed with 2 ml developing reagent in a test tube. The developing mixture comprised 50 parts BCA reagent A (1% (w/v) bicinchoninic acid, 2 % (w/v) $Na_2HCO_3.H_2O$, 0.16 % (w/v) Na_2 tartate, 0.4 % (w/v) NaOH, 0.95 % (w/v) $NaHCO_3$, pH 11.25) to 1 part BCA reagent B (4 % (w/v) $CuSO_4$). The test tube was incubated at 37 °C for 30 min. 1 ml of the final solution was then transferred to a cuvette and the absorbance at 562 nm was determined. Protein concentration was determined by comparing sample absorbances to a standard curve constructed using standards of known concentration. Standards contained BSA at concentrations from 0 - 1200 µg. The standard curve and sample protein concentrations were calculated automatically by a program installed in the spectrophotometer.

2.1.4 Assays

2.1.4.1 Unpurified amylase activities

A modified Bernfeld [1955] assay was used. 100 µl of enzyme sample was incubated at 80 °C with 425 µl of buffer (50 mM Tris, 5 mM EDTA, 6.7 mM NaCl, pH 8.5 at 20 °C, pH 8 at 80 °C), 150 µl of substrate (50 mM Tris, 5 mM EDTA, 6.7 mM NaCl, 1 % (w/v) soluble potato starch, pH 8.5) and 75 µl of 2 mM $CaCl_2$ for 20 min. The reaction was then stopped with 350 µl developing solution (1 % (w/v) DNSA, 0.4 M NaOH) and boiled for 5 min to develop the assay (colour change, yellow to brown). The assay solution was then cooled on ice for 5 min and the A_{550} was measured.

A calibration curve was made by substituting differing concentrations of maltose for the enzyme sample (Appendix I). Blanks contained distilled water in place of the enzyme sample. One unit of enzyme activity was defined as 1 µmole of reducing sugars liberated per min under assay conditions.

2.1.4.2 Amylase

Conditions were identical to those described in section 2.1.4.1 except the enzyme sample was incubated with 650 µl of substrate (50 mM Tris, 1 % (w/v) soluble potato starch, 6.7 mM NaCl, 2 mM $CaCl_2$, pH 8.5).

2.1.4.3 Pullulanase

Conditions were identical to those described in section 2.1.4.1 except the enzyme sample was incubated with 650 µl of substrate (50 mM Tris, 1 % (w/v) Pullulan, 6.7 mM NaCl, 2 mM $CaCl_2$, pH 8.5).

2.1.5 Polyacrylamide gel electrophoresis (PAGE)

2.1.5.1 SDS-PAGE

Proteins were analysed by SDS-PAGE. 12 ml of resolving gel solution (10 % (w/v) acrylamide, 0.375 M Tris, pH 8.8, 0.1 % (w/v) SDS) was polymerised by adding 50 µl of 10 % (w/v) ammonium persulphate and 12.5 µl of TEMED. This solution was then poured into a mini-gel apparatus (ATTO corporation, Nagoya, Japan), overlaid with water saturated butanol and left to set. When set the butanol was poured off and the gel surface was washed with deionised water. A 6.9 ml volume of stacking gel solution (5 % (w/v) acrylamide, 0.13 M Tris, pH 6.5, 0.1 % (w/v) SDS) was polymerised by adding 50 µl of 10 % (w/v) ammonium persulphate and 10 µl of TEMED. This solution was poured over the resolving gel in the gel chamber. Wells were formed by inserting a well-forming comb into the top of the gel chamber before the stacking gel formed. When the acrylamide had polymerised the gel apparatus was placed in the electrophoresis tank. The upper and lower reservoirs of the tank were filled with tank buffer (50 mM Tris, 0.4 % (w/v) glycine, 0.1 % (w/v) SDS) prior to removal of the well-forming comb.

Protein samples were prepared by adding an equal volume of sample buffer (0.125 M Tris, pH 6.8, 4 % (w/v) SDS, 20 % (w/v) sucrose, 10 % (v/v) β-mercaptoethanol, 0.008 % (w/v) bromophenol blue) and boiling for 2 - 3 min. Electrophoresis through the stacking gel was carried out with a current of 10 mA, and through the resolving gel at 20 mA. The electrophoresis was stopped when the dye front reached the end of the gel. The gels were stained with 0.5 % (w/v) Coomassie brilliant blue R in methanol:acetic acid:water (9:2:9 (v:v:v)) and destained with methanol:acetic acid:water (2:1:7 (v:v:v)).

2.1.5.2 Native-PAGE

Native polyacrylamide gel electrophoresis was identical to SDS-PAGE, except SDS and β-mercaptoethanol were absent in all buffers.

2.1.6 Preparation of Affinity Chromatography Matrix

Sepharose 6B-α-cyclodextrin was synthesised from epoxy-activated Sepharose 6B and α-cyclodextrin by the method of Vretblad [1974]. 5g of freeze-dried epoxy-activated sepharose 6B was reconstituted in distilled water for 1 h. The distilled water was drained off and replaced with 0.1 M NaOH and any excess liquid was removed by aspiration. The matrix was reacted with 4 g of α-cyclodextrin dissolved in 12 ml of 0.1 M NaOH by incubation for 16 h at 45 °C in a shaking incubator.

The matrix was washed with distilled water for 30 min, 2.5 % (w/v) glucose for 30 min and again with distilled water for 30 min on a glass filter. Finally, the matrix was washed with 20 mM Tris-HCl (pH 8.0) for 2 h prior to packaging into a column (Econo column, 200 x 10 mm, Biorad, Hercules CA, USA).

2.2 MOLECULAR BIOLOGY MATERIALS AND METHODS

2.2.1 Enzymes, reagents and other materials

Chloroform, magnesium sulphate, potassium acetate, sodium acetate, tri-sodium citrate were of the highest quality and were obtained from Fisons, Loughborough, Leicestershire, UK.

[α^{32}P] dCTP, [γ^{32}P] dATP, blocking agent, Hybond-N$^+$ membranes and Hyperfilm™ MP (High performance autoradiograph film) were supplied by Amersham, Little Chalfont, Buckinghamshire, UK. Bacto-agar, Bacto-tryptone and Bacto-yeast extract were supplied by Difco laboratories, Detroit MI. USA. Biotaq® and Biotaq® reaction buffers were supplied by Bioline, London, UK. Designed primers were supplied by Perkin-elmer Applied Biosystems UK, Warrington, Cheshire, UK. Dextran sulphate was supplied by USB, Cleveland OH, USA. DNA molecular weight markers were supplied by Gibco BRL, Grand Island NY, USA. Geneclean® and MERmaid® DNA extraction kits were supplied by Bio101, Vista CA, USA. G-50 Sephadex matrix, M13 sequencing and reverse primers and pUC18 plasmid vector were supplied by Pharmacia, Uppsala, Sweden. IPTG and X-gal were supplied by Calbiochem, La Jolla CA, USA. JM109 High competent cells (*Eschericia coli,* e14$^-$(McrA$^-$) *recA1 endA1 gyrA96 thi-1 hsdR17* (r$_K^-$ m$_K^+$) *supE44 relA1* Δ(*lac-proAB*) [F' *traD36 proAB lacI*^{q}ZΔ*M15*]) and pGEMT vector system were supplied by Promega. λ MAXI DNA preparation kit was supplied by Qiagen, Hilden, Germany. Lithium chloride was supplied by Acros, Geel, Belgium. Restriction endonucleases and their reaction buffers were supplied by New England Biollabs, Beverly MA, USA. SeaKem® LE agarose was

supplied by FMC BioProducts, Rockland ME, USA. T4 DNA ligase and T4 polynucleotide kinase were supplied by Boehringer-Mannheim, Mannheim, Germany. XL1-Blue MRA(P2) cells (*E. coli*, Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 gyrA96 relA1 lac* (P2 lysogen)) were supplied by Strategene, La Jolla CA, USA.

All other substrates and reagents used in molecular biology methods throughout this work were purchased from Sigma, St. Louis MO, USA.

2.2.2 Preparation of gDNA from *T. natronophilum*

2.2.2.1 Large scale preparation

Cells could be used freshly prepared or from frozen stocks. Cell pellets were resuspended in 10 ml distilled water per gram of wet weight. Resuspended cells were incubated at 37 °C for 1 h in the presence of lysozyme (30 mg predissolved in 1 ml saline-EDTA pH 7.0 per 10 ml suspension) and proteinase K (3 mg per 10 ml suspension). Complete lysis was brought about by the addition of 0.4 ml 10 % SDS per 10 ml and a further 30 min incubation at 37 °C. Lysed cells were shaken with one half volume of chloroform/isoamyl alcohol (24:1 (v:v)) for 15 min. The suspension was then dispensed in 1 ml aliquots in to 1.6 ml microfuge tubes and centrifuged at 12,000 x *g* for 5 min. The upper aqueous phase was recovered and protein extraction using chloroform/isoamyl alcohol (24:1 (v:v)) was repeated until the absence of a milky precipitate at the solvent interface was observed. Two volumes of ice cold ethanol were added to the aqueous phase and the DNA present was spooled out on heat-sealed Pasteur pipettes. The DNA was then redissolved in 0.1 x standard saline citrate (0.015 M NaCl, 0.0015 M trisodium citrate, pH 7.0). Residual RNA was removed by incubation of the solution at 37 °C for 1 h with RNAse A (0.5 mg per 10 ml). A final chloroform/isoamyl alcohol (24:1 (v:v)) extraction was performed to remove the RNAse A. The DNA was precipitated with the addition of two volumes of ice cold isopropanol and was spooled onto heat-sealed Pasteur pipettes. The DNA was redissolved in deionised water and the DNA was stored over a drop of chloroform at 4 °C.

2.2.2.2 Small scale preparation

Approximately 100 mg of *T. natronophilum* cell paste was resuspended in 567 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) by repeated pipetting. 30 µl of 10 % (w/v) SDS and 3 µl of Proteinase K (20 mg/ml) were added to the cell suspension to give a final concentration of 100 µg/ml Proteinase K and 0.5 % (w/v) SDS. The solution was thoroughly mixed and incubated at 37 °C for 1 h. 100 µl of 5 M NaCl was added and the tube contents mixed prior to the addition of 80 µl CTAB/NaCl solution (10 % (w/v) CTAB in 0.7 M NaCl).

The resulting mixture was thoroughly mixed and incubated at 65 °C for 10 min. An equal volume (700 - 800 μl) of chloroform:isoamyl alcohol (24:1 (v:v)) was added to the milky white mixture and the tube contents were mixed, then centrifuged at 12,000 x *g* in a microcentrifuge. The upper aqueous layer was transferred to a fresh, sterile 1.5 ml microfuge tube and an equal volume (600 - 700 μl) of phenol: chloroform:isoamyl alcohol (25:24:1 (v:v:v)) was added and the tube contents were mixed prior to centrifugation at 12,000 x *g* in a microcentrifuge. Again the upper aqueous layer was transferred to a fresh, sterile 1.5 ml microcentrifuge tube and placed on ice.

2.2.3 Ethanol precipitation of DNA

Ethanol precipitation was performed when DNA needed to be concentrated or the solvent needed to be exchanged. One tenth volume of 3 M sodium acetate (pH 5.2) and two volumes of absolute ethanol (stored at -20 °C) was added to the DNA sample. This was incubated at -20 °C or below for at least 30 min and DNA was recovered either by spooling on to heat-sealed Pasteur pipettes or by centrifugation at 12,000 x *g* for 10 min in a microcentrifuge, depending on the size of the DNA in question. The DNA was then washed by adding a small volume (approximately 500 μl) of 70 % (v/v) ethanol (stored at -20 °C) and gently inverting the 1.5 ml Microcentrifuge tube. The DNA was recovered by centrifugation at 12,000 x *g* for 2 - 5 min in a microcentrifuge. The pellet was then air dried and resuspended in the desired volume of buffer.

2.2.4 DNA Estimation

Purity of a DNA sample was determined by calculating the A_{260}/A_{280} ratio, a figure of 1.8 denoting that the sample is pure.

The concentration of DNA in a solution was determined by measuring the absorbance at 260 nm [Sambrook *et al.* 1989]. An absorbance of 1 unit at 260 nm equates to a dsDNA concentration of 50 μg/ml.

2.2.5 Agarose Gel Electrophoresis

Solutions containing DNA were examined by agarose gel electrophoresis; this allows the separation of linear, duplex DNA of differing sizes between 0.8 - 10 kb [Sambrook *et al.* 1989]. Agarose was dissolved in an appropriate amount of 1x TAE buffer (40 mM Tris-

acetate, 1 mM EDTA) by heating. The solution was then cooled to approximately 45 °C prior to the addition of ethidium bromide to final concentration of 0.5 μg/ml. This was then quickly poured into a prepared perspex gel mould. A well-forming comb was then placed into position and the gel was left to set at room temperature on a flat even surface. Once set, the gel was placed in a gel tank and covered with 1x TAE and the well-forming comb was removed. Samples were prepared by adding an appropriate amount of 6x loading buffer (0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol, 40 % (w/v) sucrose). The samples were then loaded into the wells and the gel was electrophoresed at a constant voltage of 60 - 75 V until the xylene cyanol dye front was 1 cm from the bottom edge of the gel. The DNA bands were then visualised using a UV transilluminator.

2.2.6 Restriction digestion of DNA

Digestion of DNA using endonucleases was carried out as specified in the manufacturer's instructions. The DNA was incubated with the enzyme in buffer (supplied with the enzyme as a 10x concentrate) at 37 °C. For digestion with two endonucleases, the enzymes could be used simultaneously if the buffer requirements were similar. If this was not the case, however, the enzymes were used sequentially, digesting first with the enzymes requiring the lowest buffer concentration. The buffer concentration was then adjusted to suit the second reaction and the enzyme added. Alternatively, the DNA was ethanol precipitated after the first incubation and resuspended in the second reaction buffer. Incubation times varied from 1 h to overnight.

2.2.7 Amplification of DNA - Polymerase chain reaction (PCR)

Reactions generally contained approximately 100 ng of DNA, 100 μmol of each primer, and 10 mM each of dATP, dCTP, dGTP, and dTTP in a final volume of 50 μl. The reactions were carried out using the Biotaq® enzyme and were buffered with 10x (NH_4) solution (160 mM $(NH)_4SO_4$, 670 mM Tris-HCl (pH 8.8 at 25 °C), 0.1 % (v/v) Tween-20). The reaction mixture was overlaid with 30 μl of mineral oil to prevent evaporation. Reactions were incubated for 94 °C prior to initiation of the PCR. Amplification was carried out as follows: denaturation at 94 °C for 45 s, annealing at 50 °C for 2 min, and extension at 72 °C for 1 min. A Ceteus DNA Thermal Cycler (Perkin-Elmer, Norwalk CT, USA) was used to amplify DNA fragments. A 10 μl sample was then visualised on a 2 % (w/v) agarose gel.

2.2.8 Preparation of plasmid DNA

2.2.8.1 Mini prep - alkaline lysis method

1.5 - 3ml of an overnight culture was pelleted at 12,000 x g in a microcentrifuge for 2 min. The medium was removed by aspiration, leaving the bacterial pellet as dry as possible. The cell pellet was then resuspended in 100 µl of ice-cold miniprep lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0), and incubated for 5 min at room temperature. 200 µl of a freshly prepared solution containing 0.2 M NaOH, 1 % (w/v) SDS was added and mixed by inversion. This was incubated on ice for 5 min. 150 µl of ice-cold 3 M potassium acetate solution, pH 4.8, was added, mixed by inversion and incubated on ice for 5 min. The mixture was then spun down at 12,000 x *g* in a microcentrifuge for 5 min and the supernatant transferred to a fresh tube. 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1 (v:v:v)) was added, mixed by vortexing for 1 min and centrifuged at 12,000 x *g* for 2 min. The upper, aqueous phase was transferred to a fresh tube and 1 volume of chloroform:isoamyl alcohol (24:1 (v:v)) added and mixed as before. The aqueous phase was again transferred to a fresh tube and ethanol precipitated, as previously described. The DNA pellet was resuspended in 50 µl deionised water and 0.5 µl 10 mg/ml RNAse A added and incubated for 5 min at room temperature.

2.2.8.2 Maxiprep - alkaline lysis method

E. coli cells containing the plasmid of interest were cultured overnight, at 37°C, in a 100 ml volume of LB medium containing 100 µg/ml ampicillin. The cells were centrifuged at 5,000 x *g* for 20 min and the cell pellet was resuspended in a 5 ml volume of ice-cold lysis buffer (25 mM Tris-HCl, pH 7.5, 50 mM glucose, 1 mM EDTA). A 10 ml volume of 0.2 M NaOH containing 1 % (w/v) SDS was added and the cells gently agitated to promote cell lysis. Sodium acetate was added to the lysed cells to a concentration of 1 M (from a 3 M solution, pH 5.2) and the mixture incubated on ice for 30 min. The precipitated material was removed by centrifugation at 5,000 x *g* for 10 min. The supernatant was then mixed with 25 ml of ice-cold isopropanol and incubated on ice for 30 min. This mixture was then centrifuged at 5,000 x *g*. The pellet was dissolved in 2ml of 1x TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and 2.5 ml of ice-cold 5 M LiCl was added. After incubating on ice for 5 min, the mixture was centrifuged at 5,000 x *g* for 10 min, the supernatant removed, and the DNA precipitated with absolute ethanol as described in section 2.2.3.

The DNA pellet was air-dried and resuspended in 0.5 ml 1x TE buffer containing 0.25 mg RNase A from a 10 mg/ml solution. After incubating at room temperature for 30 min, the DNA was precipitated by the addition of an equal volume 1.6 M NaCl, 13 % (w/v) PEG 8000 and pelleted by centrifugation at 12,000 x *g* for 5 min. The pellet was resuspended in

0.5 ml of 1x TE buffer and extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 (v:v:v)). The plasmid DNA was then precipitated (section 2.2.3) and resuspended in 100 μl of deionised water.

2.2.9 Geneclean® and MERmaid® methods

The Geneclean® and MERmaid® methods use silica-based matrices called Glassmilk® and Glassfog®, respectively, that have a high affinity for DNA. The DNA was purified from agarose as per manufacturer's instructions.

2.2.10 Dephosphorylation of oligonucleotides

Dephosphorylation of 5' cohesive ends was achieved using Shrimp Alkaline Phosphatase (SAP). 4 μl of 10x SAP reaction buffer (supplied by the manufacturer) was added to the restriction digested pDNA (2μg in 36 μl) in a 0.6 ml microfuge tube and the contents were gently mixed. The SAP was then added to a final concentration of 1 U/10 μl. Once mixed, the mixture was incubated at 37 °C for 15 min. SAP was inactivated by incubating the reaction mixture at 65 °C for 10 min. To ensure the isolation of the DNA from any possible SAP activity, remaining the whole DNA sample was electrophoresced on a 1 % (w/v) agarose gel and the relevant band excised and subjected to extraction using the Geneclean® kit.

2.2.11 Ligations

Ligation of cohesive ends was carried out using 1 unit of T4 DNA ligase (supplied at a concentration of 1 unit/μl) in the buffer supplied with the enzyme. A ratio from 1 - 3 to 1 of vector with respect to insert was used in a total volume of 10μl. The reaction was incubated overnight at 15 °C or at room temperature for 3 h. The whole reaction mixture was used to transform competent *E. coli* cells.

2.2.12 DNA Sequencing

DNA sequencing was automated and was performed in house. The DNA sequencing is based on the method of Sanger *et al.* [1974], where the extension of a complementary strand is terminated with the insertion of dideoxy nucleotide (ddNTP). As each ddNTP is

specifically labelled with one of four fluorescent dyes, it was not necessary to perform 4 separate reactions. The products from the single reaction were analysed on a polyacrylamide gel using an ABI PRISM™ 377 DNA sequencer (Applied Biosystems, Fostercity CA, USA)

2.2.13 Transformation of DNA into bacteria

50 µl of JM109 high competence cells were used for each sample to be transformed. The cells were slowly thawed by placing on ice for 5 min. Once thawed, 1 - 10 µl of a ligation reaction or control was added to the cells in a sterile 1.6 microfuge tube and the contents were gently mixed using a pipette tip. The mixture was then stored on ice for 30 min, prior to heat-shock at 42 °C for 2 min, after which the cells were immediately placed on ice for 2 min. Each transformation reaction was then transferred to a sterile 15 ml Falcon tube containing 950 µl of sterile LB broth and incubated in a shaking incubator (~150 rpm) for 1 h at 37 °C. Following incubation the cells were dealt with as according to section 2.2.14.

2.2.14 Selection of transformed cells

2.2.14.1 Antibiotic selection

200 µl of transformed cells were spread on to an LB agar plate supplemented with 100 µg/ml ampicillin and incubated overnight at 37 °C in a plate incubator.

2.2.14.2 Blue/white selection

200 µl of transformed JM109 cells were spread on to an LB agar plate supplemented with 100 µg/ml ampicillin, 0.1 mM IPTG and 40 µg/ml X-gal.

2.2.15 Southern blotting of DNA from agarose gels to nylon membranes

The DNA sample was size separated by agarose gel electrophoresis, as described in section 2.2.5. Following electrophoresis, the gel was incubated for 45 min with gentle agitation in denaturation solution, consisting of 0.5 M NaOH and 1.5 M NaCl. The gel was then soaked in 0.25 M HCl for 15 min. The gel was then briefly rinsed with distilled water prior to being incubated for 30 min with gentle agitation in neutralisation solution, which contains 1 M Tris (pH 7.4) and 1.5 M NaCl. During this 30 min incubation the solution was replaced twice. The Southern blot was then prepared as follows. An inverted gel casting

tray was used as a raised platform with a shallow dish, filled to a depth of 2.5 cm with 20x SSC buffer. A strip of Whatman 3MM paper was laid over the platform, to serve as a wick, onto which the gel, membrane and two pieces of Whatman 3MM paper were placed. The filter and the Whatman 3MM paper had been previously cut to the same size as the gel and pre-wetted with 2x SSC buffer. Bubbles were removed at each stage using a sterile Pasteur pipette to promote even transfer of DNA. Capillary transfer of buffer through the gel, membrane and Whatman 3MM papers was then effected by placing a 5 - 8 cm stack of paper towels on top of the Whatman 3MM papers. A glass plate was then placed onto the stack and pressure was applied to the stack by placing a 500 g weight onto the glass plate. The apparatus was left overnight. The following day, the stack of paper towels was replaced and the apparatus was left for a further 4 h. The apparatus was dismantled, but care was taken to keep the membrane and gel together. Using a soft pencil or a ball point pen the well positions on the gel were marked onto the membrane for future reference. The gel was then carefully peeled away from the membrane and discarded. To remove any agarose, the membrane was soaked in 6x SSC buffer for 5 min. The membrane was then left to air-dry on paper towels prior to fixing the transferred DNA to the membrane by baking at 80 °C for 1 - 2 h.

2.2.16 Denaturation of DNA bonded to nylon membranes

Once the DNA to be screened had been transferred to nylon membranes it was denatured to allow the hybridisation of probes. Three trays containing 3MM Whatman paper cut larger than the membranes were saturated with (1) Denaturation solution, 0.2 M NaOH, 1.5 M NaCl, (2) Neutralisation solution, 0.4 M Tris-HCl, pH 7.6 and (3) 2x SSC. Using forceps, each membrane (DNA side up) was incubated in trays 1 - 3, for 7, 7 and 2 min respectively. Care was taken whilst transferring from one tray to the next to minimise the transferral of solutions by dragging the membrane along the top of the lip of the trays when carrying over to the next tray. After the final incubation, the membrane was left on paper towels to air dry. Finally, the denatured DNA was fixed to the membrane by incubating in an oven at 80 °C for 1 - 2 h.

2.2.17 Hybridisation of membranes from screening and Southern blots

The DNA of interest was transferred to Hybond-N^+ membranes as described in sections 2.2.15 and 2.2.19.2 and treated as per section 2.2.16. Each membrane was incubated in 5ml pre-hybridisation solution consisting of 5 % (w/v) Dextran sulphate, 0.5 % (w/v) Blocking agent, 0.1 % (w/v) SDS in 5x SSC (50 µl of freshly denatured Salmon teste DNA was added to 5 ml of pre-hybridisation mixture before use), at 45 °C for 2 h in a rotating hybridisation

oven, Biometra OV4 (Biometra, Göttingen, Germany). After the pre-hybridisation, 2 ml of pre-hybridisation solution was removed for each membrane and 5 µl of probe added for each membrane. Hybridisation was carried out at 45 °C, unless otherwise stated, and incubated overnight for approximately 16 h.

The following morning, the hybridisation solution was disposed of and the membranes were washed for 20 min once in 25 ml of 1x SSC, 0.5 (w/v) SDS and twice in 25 ml of 0.5x SSC, 0.5 % (w/v) SDS, at 45 °C, unless otherwise stated. The washed membranes were then wrapped in plastic food wrap and exposed to x-ray film in an x-ray cassette. The cassette was stored at -70 °C for 24 - 48 h. The autoradiographs were developed using a Hyperprocessor (Amersham, Little Chalfont, Buckinghamshire, UK).

2.2.18 Removal of un-incorporated radioactive dNTPs

The labelled probe was separated from un-incorporated [γ^{32}P] dATP or [α^{32}P] dCTP by passing through a 1 ml Sephadex G-50 column. This was prepared in a 1 ml syringe barrel, that had been plugged with silane-treated glass wool. The Sephadex G-50 storage buffer was removed by centrifugation at 3,000 x *g* for 2 min and the matrix was washed with 1x TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The column was centrifuged at 3,000 x *g* for 2 min to remove traces of liquid. The labelling mixture was applied to the top of the column and the labelled oligonucleotide separated from un-incorporated [γ^{32}P] dATP by a final centrifugation step (3,000 x *g* for 2 min) into a 1.5 ml sterile microcentrifuge tube. The probe was denatured by heating to 100 °C for 10 min prior to hybridisation.

2.2.19 Screening procedures

2.2.19.1 Preparation of top-agar plates for screening gDNA λ library

A single colony of XL1 Blue MRA cells (P2) was used to inoculate 20 ml of LB supplemented with 10 mM $MgSO_4$ and 0.2 % (w/v) maltose in a sterile 50 ml conical tube and incubated in a shaking incubator at 37 °C until an OD_{600} of 1.0. The P2 cells were immediately pelleted at 1,500 x *g* for 10 min. The pellet was resuspended in ice-cold 10 mM $MgSO_4$ to give a final OD_{600} of 0.5. The cells were stored on ice until needed (can be stored at 4 °C for up to 24 h). 1 - 10 µl of λ library stock was used to infect 200 µl of P2 cells per plate to be screened. The mixture contained in a sterile 15 ml conical tube was incubated at 37 °C for 20 min with occasional agitation. 3 ml of molten top-agar (LB broth supplemented with 0.7 % (w/v) agarose) at 48 °C was added to each 15 ml conical tube prior to mixing by inversion and immediately pouring on to pre-warmed agar plates (37 °C).

Once set, the plates were incubated at 37 °C overnight in a plate incubator.

2.2.19.2 Transfer of λ clone DNA from top-agar plates to nylon membranes

Each plate and membrane were numbered to facilitate identification at a later point, and this was achieved using a soft pencil to mark the delicate membrane. The membrane was gently lowered, centre first, on to the surface of the top-agar plate, taking care not to get any air bubbles trapped beneath it. Once in position, the membrane and agar were stabbed in a specific way (near to the edge of the membrane) using a syringe and hyperdermic needle primed with water-resistant ink, so that plate and autoradiograph could be re-aligned at a later point. After 1 - 2 min, the membrane was carefully removed using a pair of forceps and left DNA side up on paper towels to air dry for 15 min.

2.2.19.3 Obtaining λ clones from screened plates

Once a positive clone was identified from the autoradiographs and the corresponding plaque found, a plaque plug was taken. A sterile pasteur pipette is lowered on to the plaque in question so that a "plug" of agar containing that single plaque is removed from the plate. The plug was expelled from the pasteur pipette in to a sterile 1.6 ml microfuge tube containing 1 ml of SM buffer (0.58 % (w/v) NaCl, 0.2 % (w/v) $MgSO_4 \cdot 7H_2O$, 50 % (v/v) 1 M Tris-HCl (pH 7.5), 2 % (w/v) Gelatin solution) and a single drop of chloroform. The tube was then set on a vertical rotating platform and gently inverted at 4 °C for 3 - 4 h. The resulting suspension can be stored indefinitely at 4 °C and was used either to culture the single λ clone or to perform a second screen.

2.2.19.4 Liquid culture of λ clones

A single P2 colony was used to inoculate 100 ml of NZCYM medium (1 % (w/v) NZ amine, 0.5 % (w/v) NaCl, 0.5 % (w/v) Bacto-yeast extract, 0.2 % (w/v) $MgSO_4 \cdot 7H_2O$, 0.1 % (w/v) Casamino acids, pH 7.0) in a 500 ml conical flask and incubated overnight at 37 °C with vigorous shaking (~300 rpm). The following morning, the OD_{600} was measured and the cell concentration calculated, assuming that OD_{600} 1.0 ≡ 8×10^8 cells/ml. Aliquots containing 10^{10} cells were removed for each λ clone to be amplified and pelleted in 15 ml conical tubes by centrifugation at 1,500 x *g* for 10 min at room temperature. Each pellet was resuspended in 3 ml of SM buffer and 10 - 50 µl of plaque plug suspension was added to each aliquot and incubated at 37 °C with occasional agitation. Each preparation was then added to 500 ml of prewarmed NZCYM medium (37 °C) in 2 L conical flasks. The flask were than incubated at 37 °C with vigorous shaking overnight. The following day, 10 ml of chloroform was added to the lysed cultures and incubated for a further 10 min. The λ clone DNA was then prepared as outlined in section 2.2.19.5.

2.2.19.5 λ Clone DNA isolation and preparation

A MAXI λ DNA preparation kit was used to prepare DNA from cultured λ bacteriophage. The kit was used as per manufacturer's instructions.

CHAPTER 3

Purification of amylolytic activities from *Thermopallium natronophilum*

3.1 INTRODUCTION

Initial studies on *T. natronophilum* indicated that the micro-organism possessed the ability to hydrolyse starch [Gist-brocades internal report], although it was not clear which starch hydrolysing enzyme was responsible. In order for further investigations to be carried out, the hydrolase needed to be purified. This chapter deals with the preparation of the enzyme from cell extracts.

3.2 MATERIALS

Growth of *T. natronophilum* cells took place at CAMR (Centre for Applied Microbiology Research) at Porton Down, Salisbury, under the supervision of Prof Richard Sharp and Dr Neil Raven. The growth conditions and requirements of the organism are outlined in Section 2.1.2. Superdex 200 (HR 16/60) and Mono Q (HR 5/5) columns were connected to a Fast Protein Liquid Chromatography (FPLC) system and were supplied by Pharmacia, Uppsala, Sweden. The α-cyclodextrin-6B-sepharose affinity column was prepared as described in 2.1.6.
Other materials and their suppliers mentioned in this chapter are listed in chapter 2.

3.3 METHODS

3.3.1 Protein Preparation

The cell paste (1 - 2 g) was diluted to 0.2 g/ml with extraction buffer (50 mM Tris-HCl, 5 mM EDTA, pH 8.5). The mixture was then sonicated at 3 x 10 s bursts at 16 - 18 microns, peak to peak (3 mm probe, 150 W Ultrasonic Disintegrator Mk 2, MSE, Crawley, UK). Cellular debris was removed by centrifugation at 47,800 x *g* for 60 min at 4 °C (Beckman, RC-5B, Sorvall, rotor SS-34). The supernatant was decanted and stored on ice. The pellet was washed and resuspended in a few mls of breaking buffer and recentrifuged. The supernatant was decanted and combined with the first supernatant.

The cell-free extract was concentrated using a Centriprep-30 unit (Amicon, Beverly MA, USA) to about 1.5 - 2 ml and the filtrate was discarded, as there was no associated amylase or pullulanase activity. The concentrated protein sample was subjected to gel filtration on a HR 16/60 Superdex 200 column. Protein was eluted using 20 mM Tris-HCl buffer (pH 8.5) at a flow rate of 1 ml/min. The fractions possessing amylase and pullulanase activity were pooled prior to anion-exchange chromatography. Anion-exchange chromatography was achieved using a HR 5/5 Mono Q column and protein was eluted with a salt gradient of 0 - 2 M NaCl in 20 mM Tris-HCl (pH 8.5). The fractions were assayed for amylase and pullulanase activity; both peaks of activity were individually bulked and dialysed against 20 mM Tris-HCl (pH 8.5) buffer at 4 °C. The pullulanase peak resolved by anion-exchange chromatography was subjected to affinity chromatography. Affinity chromatography was achieved using a column packed with α-cyclodextrin-Sepharose 6B matrix in conjunction with an FPLC machine. The sample was applied to the column and unbound protein was eluted using 20 mM Tris-HCl (pH 8.5); bound protein was eluted from the column with the application of 20 mM Tris-HCl (pH 8.5); containing 0.2 %(w/v) α-cyclodextrin. The fractions were then assayed for pullulanase activity. The pullulanase activity from the affinity column was subjected to anion-exchange chromatography on Mono-Q and protein was eluted using a shallower gradient than used previously. The amylase peak from anion-exchange was applied a second time to Mono Q and protein was eluted using a shallower gradient formed by the previously mentioned buffers. The fractions were assayed for amylase activity.

3.3.2 Activity Staining

Native polyacrylamide gels containing the proteins of interest were incubated in several volumes of 50 mM Tris-HCl, 1 % (w/v) starch, 6.7 mM NaCl, 2 mM $CaCl_2$ (pH 9.0) at 85 °C for 90 minutes. The gel was then removed from the incubation solution and quickly rinsed with distilled water to remove excess starch before staining. Staining was performed by gently agitating the gel in iodine solution (2.5 % (w/v) HCl, 0.6 % (w/v) KI, 0.06 % (w/v) I_2) on a platform shaker. Unstained regions on the gel denoted activity.

The remaining procedures mentioned in this chapter are outlined in chapter 2.

3.4 RESULTS

3.4.1 Purification of two amylase activities from *T. natronophilum*

Gel filtration, anion-exchange chromatography and affinity chromatography were used to resolve two amylase activities from cell extracts of *T. natronophilum.* These amylase activities were subsequently characterised and classified as being an α-amylase and a type-I pullulanase (details of characterisation follow in chapter 4).

Amylase and pullulanase activities were measured at 80 °C, the same temperature of the environment from which the organism was isolated. The assays used were modified from Bernfeld [1955], which is a discontinuous colourimetric assay, involving the reaction between free reducing ends of saccharides and 3,5-dinitrosalicylic acid. The assay was validated according to (i) linearity between product concentration and absorbance at 550nm, (ii) linearity between the rate of increase of product concentration and enzyme concentration (iii) linearity between product concentration and incubation time (Appendix I).

The preparation of cell extract from whole cells retained approximately 70 - 90 % of total amylase and pullulanase activity. The specific activity of amylase and pullulanase in *T. natronophilum* cell free extracts was measured to be between 0.04 - 0.06 U/mg of protein and 0.6 - 0.8 U/mg of protein respectively.

Gel filtration was used as an initial purification step following the preparation of cell extract. Single peaks of amylase and pullulanase activity were eluted from the Superdex column at a similar elution volume (Figure 3.1). The elution profile of the activities indicated that the enzymes responsible had an approximate native molecular weight of 85 kDa, determined using a calibration curve (Figure 3.2). This initial step gave a purification factor of 1.5 for pullulanase activity, however there was a decrease in specific activity for the amylase (Tables 3.1 and 3.2). A possible explaination for a reduced specific activity may be due to the removal of calcium ions during purification. The purification data for the amylase activity are also overcast by the phenomenon that the pullulanase activity is able to hydrolyse starch in addition to pullulan.

The two activities are separated on elution from anion-exchange chromatography on Mono Q. The pullulanase and amylase are eluted from the column at NaCl concentrations of approximately 170 and 290 mM, respectively (Figure 3.3).
It was envisaged that the incorporation of an affinity step may facilitate the purification of the amylase. An α-cyclodextrin-6B-sepharose column was prepared for this purpose and has been utilised in the preparation of amylases from other organisms [Iefuji *et al.* 1996, Yamadu *et al.* 1996, Planchot and Colonna 1995]. Initial trials using cell extract revealed only

approximately half of the total amylase activity was binding to the column and subsequent observations showed that the amylase activity binding to the column was attributed to the pullulanase (results not shown). Indeed the affinity column proved to be an effective purification method for the pullulanase and once incorporated into the purification procedure, a purification factor of 566 was achieved (Figure 3.4).

Finally, both proteins were subjected to a second run on anion-exchange chromatography and protein was eluted using a shallower gradient that used on the previous application (Figure 3.5 and 3.6). The final purification factors were 1606 and 81 for the pullulanse and amylase, respectively. Indeed the purification fold value for the amylase is not an accurate representation due to the additional starch hydrolysing activity of the pullulanase activity in the early stages of purification. Using the data provided by the purification tables it would appear that the pullulanase represents approximately 0.06 % of the total protein in *T. natronophilum* cell extracts, whereas the amylase represents approximately 1.24 %.

	Volume (ml)	Total Protein (mg)	Total Activity (μmoles/min)	Specific Activity (μmoles/min/mg)	Yield (%)	Purification Fold
Cell Extract	11.2	97.8	66.7	0.682	100	-
Superdex 200	13.1	50.7	50.8	1.00	76.2	1.5
Mono Q$^{\#1}$	3.73	9.36	23.0	2.45	34.4	3.6
Affinity	10.6	0.035	13.5	386	20.3	566
Mono Q$^{\#2}$	6.0	0.005	5.47	1095	8.2	1606

Table 3.1 The purification table for pullulanase from *T. natronophilum* cell extracts

	Volume (ml)	Total Protein (mg)	Total Activity (μmoles/min)	Specific Activity (μmoles/min/mg)	Yield (%)	Purification Fold
Cell Extract	11.2	97.8	4.20	0.0429[a]	100	-
Superdex 200	13.1	50.7	1.19	0.0235[a]	28.4	-
Mono Q$^{\#1}$	2.75	3.75	0.348	0.0928	8.3	2.2
Mono Q$^{\#2}$	2.0	0.040	0.139	3.48	3.3	81.2

Table 3.2 The purification table for amylase from *T. natronophilum* cell extracts

[a] Denotes that the value represents starch hydrolytic activity of both the amylase and pullulanase.

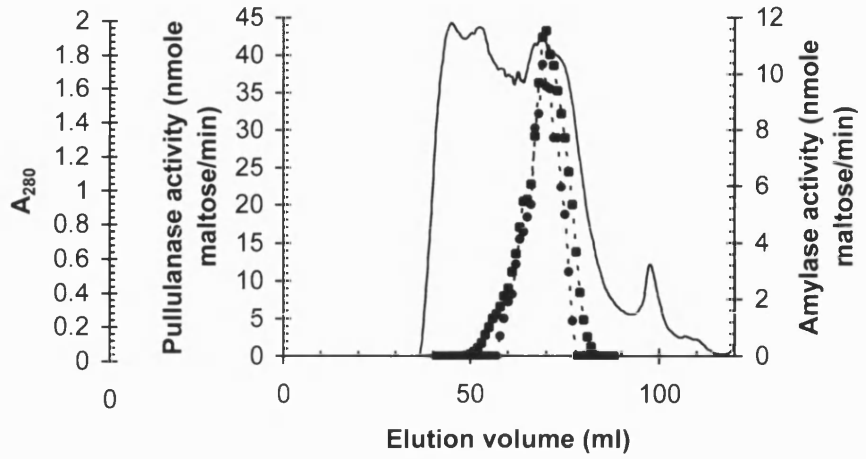


Figure 3.1 Gel filtration of *T. natronophilum* cell extract

Fractions were assayed for amylase (●) and pullulanase activity (■). Protein (—) was measured using a uv monitor linked to the FPLC system.

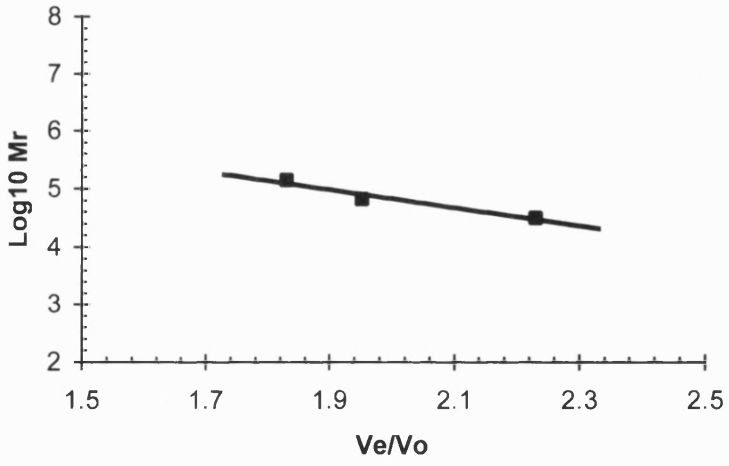


Figure 3.2 Calibration curve for S-200 gel filtration column

The protein molecular weight standards were Alcohol dehydrogenase (Yeast, 141 kDa), Serum albumin (Bovine, 66.2 kDa) and Carbonic anhydrase (Bovine, 31 kDa). The void and column volumes were 41.25 and 122 ml, respectively, determined using Blue dextran (>2000 kDa) and DNP-Lysine (367 Da).

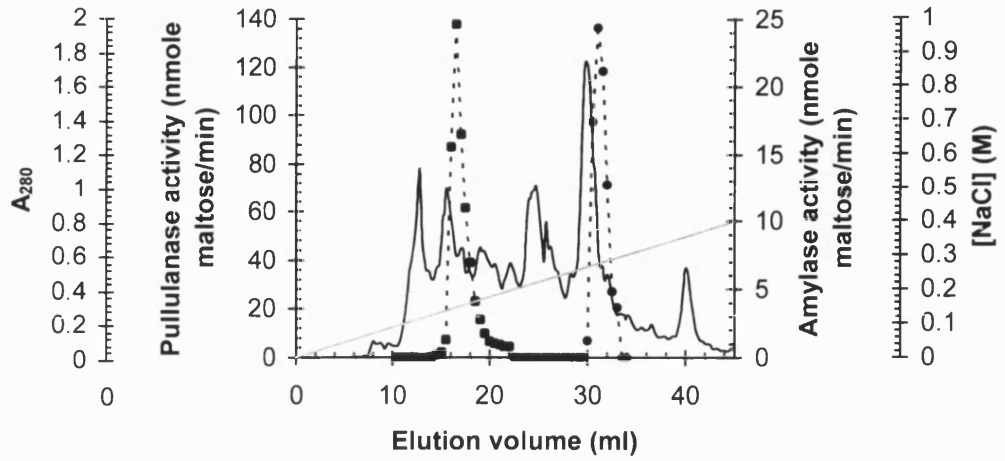


Figure 3.3 Anion-exchange chromatography of *T. natronophilum* amylase and pullulanase

Fractions were assayed for amylase (●) and pullulanase activity (■). Protein (—) was measured using a uv monitor linked to the FPLC system. Concentration of NaCl is represented by the dotted line.

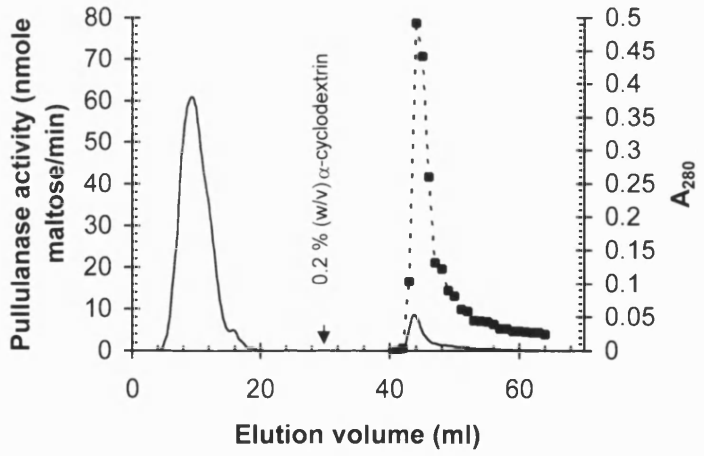


Figure 3.4 Affinity chromatography of *T. natronophilum* pullulanase

Fractions were assayed for pullulanase activity (■). Protein (—) was measured using a uv monitor linked to the FPLC system.

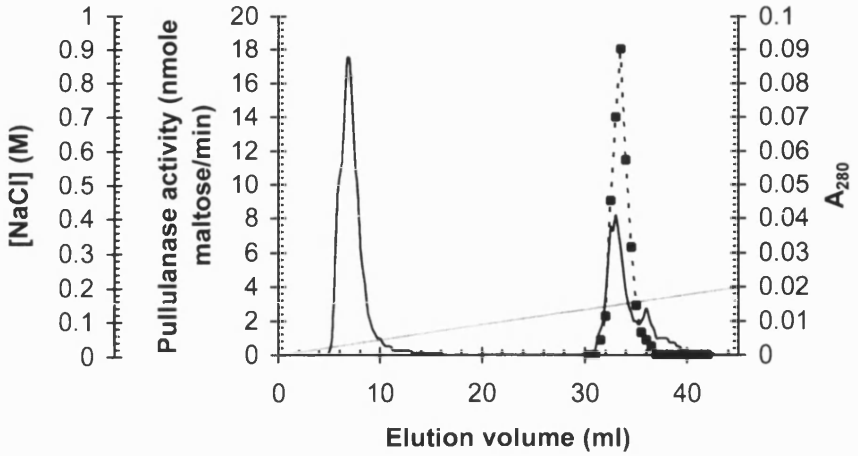


Figure 3.5 Anion-exchange chromatography of *T. natronophilum* pullulanase

Fractions were assayed for pullulanase activity (■). Protein (—) was measured using a uv monitor linked to the FPLC system. Concentration of NaCl is represented by the dotted line.

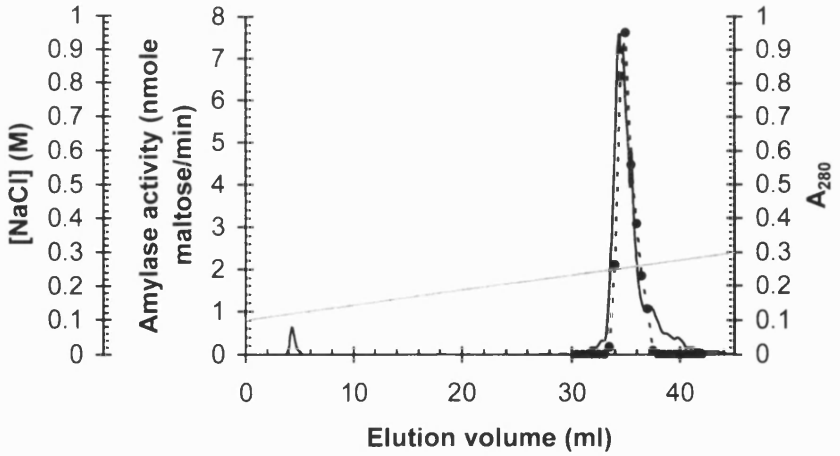


Figure 3.6 Anion-exchange chromatography of *T. natronophilum* amylase

Fractions were assayed for amylase activity (●). Protein (—) was measured using a uv monitor linked to the FPLC system. Concentration of NaCl is represented by the dotted line.

3.4.2 Analysis of amylase and pullulanase by gel electrophoresis

When the amylase and pullulnase were visualised by SDS-PAGE under reducing conditions, they appear as single bands (Figure 3.7). The amylase exhibited a slightly higher M_r of approximately 87,000 compared to that of the pullulanase which showed an approximate M_r of 83,000. When similar enzyme preparations were subjected to polyacrylamide gel electrophoresis under non-denaturing/reducing conditions there was a more pronounced difference in motility (Figure 3.8a).

3.4.3 Activity staining

Activity stains were performed in order to verify that the protein bands seen on polyacrylamide gels correlated with hydrolytic activity against starch. Activity stains on SDS-PAGE gels containing the two proteins showed no clearing zones; this is presumed to be due to the inability of the proteins to re-fold or regain activity once linearised by the addition of SDS and β-mercaptoethanol. Activity stains on native polyacrylamide gels showed two clearing zones corresponding to the two bands seen on the gel when visualised with Coomassie blue stain (Figure 3.8b)

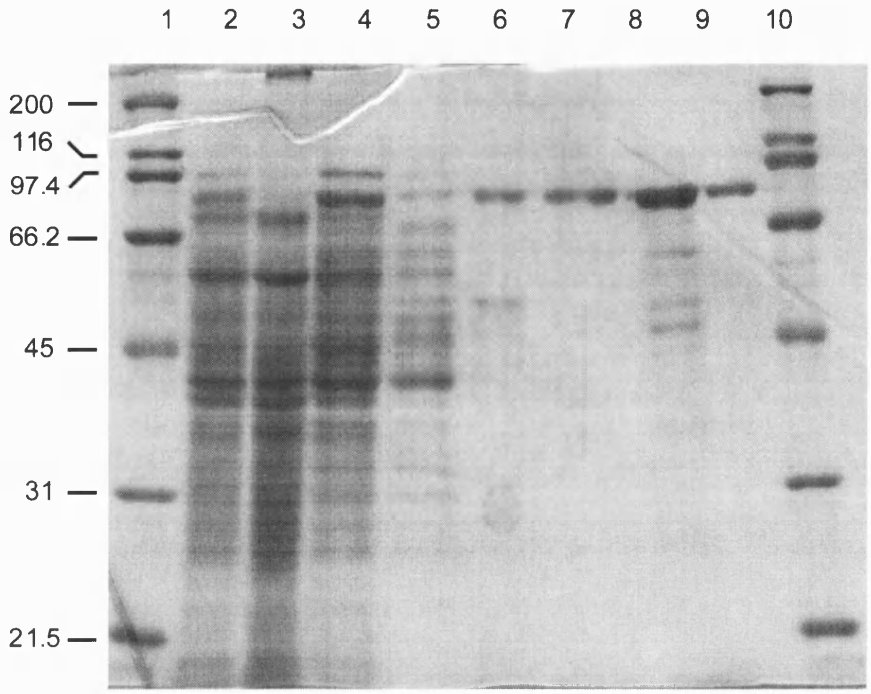


Figure 3.7 SDS-Polyacrylamide gel of a preparation of amylase and pullulanase from *T. natronophilum* cell extracts

Lanes 1 and 10: Molecular weight markers - Myosin (Rabbit skeletal muscle, 200 kDa), β-Galactosidase (*E. coli*, 116.25 kDa), Phosphorylase B (Rabbit muscle, 97.4 kDa), Serum albumin (Bovine, 66.2 kDa), Ovalbumin (Hen egg white, 45 kDa), Carbonic anhydrase (Bovine, 31 kDa), Trypsin inhibitor (Soybean, 21.5 kDa). Lane 2: Cell extract (20 μg), Lane 3: Concentrated cell extract (20 μg), Lane 4: Pooled fractions from gel filtration (10 μg), Lane 5: Pooled fractions containing pullulanase activity from Mono $Q^{\#1}$ (5 μg), Lane 6: Pooled fractions containing pullulanase activity from α-cyclodextrin-6B-sepharose affinity column (1 μg), Lane 7: Pullulanase eluted from Mono $Q^{\#2}$ (1 μg), Lane 8: Pooled fractions containing amylase activity from Mono $Q^{\#1}$ (5 μg), Lane 9: Amylase eluted from Mono $Q^{\#2}$ (1 μg).

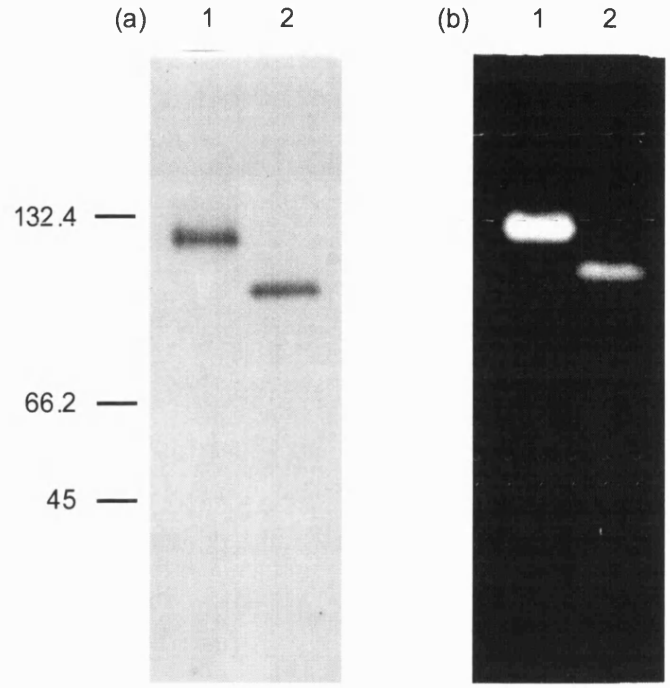


Figure 3.8 Native-polyacrylamide gel of amylase and pullulanase from *T. natronophilum*
(a) Stained with Coomassie blue. (b) Stained for starch hydrolase activity. Lane 1: Molecular weight markers - Serum albumin (Bovine, 134.4 kDa and 66.2 kDa) and Ovalbumin (Hen egg white, 45 kDa), Lane 2: Pullulanase eluted from Mono $Q^{\#2}$ (1 μg), Lane 3: Amylase eluted from Mono $Q^{\#2}$ (1 μg).

3.5 DISCUSSION

3.5.1 Purification of amylase and pullulanase from *T. natronophilum*

Starch hydrolases play an important role in the breakdown of starch into oligosaccharides. Hydrolysis of starch is essential to facilitate its assimilation into the cell. Amylose, amylopectin and indeed pullulan are macromolecular substrates (molecular weight, 250 - 2000 kDa [Drauz and Waldmann 1995]) and are unable to penetrate the 'toga'. The composition of the 'toga' in *T. natronophilum* is believed to be similar to that of *Thermotoga maritima*, which is composed of porin-type proteins [Rachel *et al.* 1990].

No activity against starch was observed when the *T. natronophilum* cell-free culture medium was assayed for amylase activity. Although the hydrolases do not seem to be exported from the cell it is thought that they are close contact with the extracellular environment. Schumann and co-workers [1991] observed that *T. maritima* cell-free culture medium contained little activity, and further studies revealed that the amylase activities were associated with the 'toga'. Other studies have also elucidated that the majority of α-amylases from micro-organisms are extracellular in nature with a minority that are intracellular or associated with the cell membrane [Abe *et al.* 1994, Koivula *et al.* 1993, Schumann *et al.* 1991]. Therefore the hydrolases are in contact or close proximity to the extracellular environment.

The fold-purification data shown in tables 3.1 and 3.2 compare well with those published for other amylases and pullulanases. Initial purification data for the amylase seems to be affected by two factors, (i) additional starch hydrolysis by pullulanase (representing approximately 50 % of total starch hydrolysis) and (ii) possible degradation of the amylase by proteolytic cleavage in the initial stages. Although there is an apparent 81-fold purification of the amylase, if the activity against starch exhibited by the pullulanase is taken into consideration, a 160-fold purification would be expected.

From the purification data it is possible to determine the percentage of the cellular protein represented by the amylase and pullulanase. The values of 1.24 and 0.06 % for the amylase and pullulanase were calculated respectively. Again these values are comparable with published data from other organisms. A relatively high value for the amylase is expected, since amylase production is enhanced in the presence of starch as a carbon and energy source. Indeed when grown in the absence of starch, levels of amylases are greatly reduced [Mc Tigue *et al.* 1994].

The specific activities of the pure amylase and pullulanase are vastly different. The specific activity of the amylase is also low when compared to other α-amylases studied, although

whether this is a factor of the enzyme's alkalophilicity (details in chapter 4) is unclear. All enzymes belonging to the α-amylase family catalyse the same basic reaction: a nucleophilic double displacement mechanism with a transient covalent intermediate, two acidic amino acids residues (Asp or Glu) of the protein being essentially involved in catalysis (i.e. one has an ionised carboxylic group, the other has an unionised carboxylic group). If this mechanism is true for the amylase from *T. natronophilum* then the low specific activity may be due to ionised carboxylic acid groups at elevated pH [Kim *et al.* 1995]. Conversely, the pullulanase has a specific activity against pullulan of approximately 1095 U/mg of protein, which is higher than any other type-I pullulanase isolated to date.

3.5.2 Molecular weight and subunit composition

The molecular weights determined by SDS-PAGE for the amylase and pullulanase are very similar. However, when the two proteins are analysed on non-denaturing gels they show a marked difference in motility. This is a reflection of a difference in size/shape/charge between the two hydrolases. As both enzymes are similar in size, as determined by gel filtration, and are monomers as determined by SDS-PAGE, this phenomenon is unlikely to be due to a difference in size. In addition, α-amylases and pullulanases belong to the $(\alpha/\beta)_8$-barrel glycosyl hydrolase family of proteins and probably share a similar tertiary structure [Janecek and Balaz 1992, Janecek 1992, 1993, 1994, 1995, 1997, Svensson 1994], thus eliminating shape. The most likely cause for the difference in motility on non-denaturing gels is the charge of the proteins, consistent with their elution from anion-exchangers at different salt concentrations.

The native subunit composition and size of the amylase and pullulanase compare well with the amylases and pullulanases studied to date. In general, α-amylases range between 22.5 - 225 kDa and can be monomeric, dimeric (homo and hetero) or hetero-trimeric. The majority are monomers with a M_r between 50 - 90 kDa, with an average of approximately 70 kDa. Pullulanases tend to be monomeric in nature and have M_r ranging between 55 -140 kDa, with an average M_r of 90 kDa.

3.6 CONCLUSIONS

T. natronophilum contains two starch hydrolases and, as amylase activity is absent from the cell-free growth medium, it would appear that they are cell bound. Taking into account that the hydrolases utilise large substrates that are unable to penetrate the "toga" it is likely that one or both are associated with the "toga".

Both enzymes appear to be monomers and have similar molecular weights, which show a good correlation with amylases and pullulanases isolated to date.

The following chapter deals with the characterisation of the two starch hydrolases and discussion of the biotechnological viability.

CHAPTER 4

Characterisation of amylolytic activities from *Thermopallium natronophilum*

4.1 INTRODUCTION

Once two amylase activities had been identified by separation on anion-exchange chromatography, the next objective was to identify and characterise the enzymes responsible. This chapter deals with the work undertaken to identify the hydrolases and to determine their basic characteristics.

For ease of explanation the two amylases are referred to as amylase I and amylase II based on the order in which they are eluted from anion-exchange chromatography.

4.2 MATERIALS

Maxamyl® and Novomyl® are amylases used commercially and were obtained from Gist-brocades, Delft, The Netherlands and Novo Nordisk, Bagsvaerd, Denmark, respectively.

The oligosaccharide analysis was carried out using an Aminex HPX-42A column (300 x 7.8 mm, Biorad, Hercules CA, USA) connected to a High Performance Liquid Chromatography (HPLC) machine (Waters 410, Waters, Milford MA, USA).

Other materials and their suppliers mentioned in this chapter are listed in chapter 2.

4.3 METHODS

4.3.1 Temperature optimum

Hydrolytic activity was measured using the standard assays in a temperature range from 60 - 105 °C. For activity measurements over 95 °C a polyethyleneglycol bath or a microtube heating block (Grant Instruments, Cambridge, UK) was used. Unpurified and purified hydrolases were studied using their respective assays.

4.3.2 pH Optimum

Hydrolytic activity was measured in a pH range between pH 6 - 10.8. The pH range was created by replacing Tris with suitable buffers (acetic acid, MES, MOPS, HEPES, diethanolamine, glycine) according to the pH required in the assay. The final pH of the assay solution was measured at the assay temperature using a pH meter.

4.3.3 HPLC Analysis

Samples were prepared by incubating 1.2 ml (0.36 U; 1U = 1 μmole reducing sugars/min) of enzyme sample with 7.8 ml of substrate buffer. Amylase I was incubated in 50 mM Tris (pH 9.5), 5 % (w/v) Zulkowsky starch and 6.7 mM NaCl. Novomyl® was incubated in 50 mM MES (pH 5.5) and 5 % (w/v) Zulkowsky starch. Amylase II was incubated in 50 mM Tris (pH 9.0), 5 % (w/v) Zulkowsky starch and 2 mM $CaCl_2$; Maxamyl® was incubated in a similar buffer at pH 7.0. Equal amounts of activity of amylase I and amylase II were incubated at 80 °C for up to 240 h, and 0.5 ml aliquots were removed and quenched on ice at regular intervals. Novomyl® and Maxamyl® were incubated at 65 °C and 70 °C, respectively, and 0.5 ml aliquots were removed and quenched on ice at regular intervals between 1 and 32 h. Identical incubations were also carried out in the absence of enzymes to assess the degree of hydrolysis due to incubation conditions.

Samples were centrifuged in a microcentrifuge at 12,000 x *g* for 5 min to remove insoluble material prior to analysis. 25 μl of sample was automatically injected into the preheated (85 °C) Aminex HPX-42A column. Degassed and filtered deionised water was used as the mobile phase and was applied at a flow rate of 0.6 ml/min. The total runtime for the column was set at 25 min. Detection of the oligosaccahrides was carried out using a RI detector unit (Waters).

4.3.4 Substrate specificity

100 μl aliquots of amylase and pullulanase samples were assayed for hydrolytic activity against amylose, amylopectin, glycogen, pullulan, starch and α, β and γ-cyclodextrins. This was achieved by assaying the enzyme samples in the usual manner in the presence of the substrate in question. The degree of hydrolysis was evaluated using the DNSA method outlined in chapter 2.

4.3.5 Thermal inactivation

Enzyme samples were dialysed against several volumes of 20 mM Tris-HCl (pH 8.5) at 4 °C for several hours. 1 ml of enzyme sample was incubated at 75, 90 and 95 °C in the absence of substrate for 1 h. 100 µl aliquots were removed at 0, 5, 10, 15, 20, 30, 40 and 60 min and immediately stored on ice. Once all the incubated samples had been collected they were assayed for hydrolytic activity against their respective assays according to section 2.1.4.

The remaining procedures mentioned in this chapter are outlined in chapter 2.

4.4 RESULTS

4.4.1 Unpurified amylase activity

Cell-free extract was obtained as previously described in section 3.2.1.

4.4.1.1 Temperature optimum

The sample used for temperature work had a specific activity of 0.005 U/mg protein and 60 µU was used in each assay. The temperature optimum for amylase activity in the cell-free extract is approximately 95 °C (Figure 4.1). However, the temperature profile is peculiar in shape and does not seem to obey the Q_{10} rule. This infers that there may be more than one amylase activity responsible, with differing temperature optima.

4.4.1.2 pH Optimum

The sample used for the pH optimum work had a specific activity of 0.01 U/mg protein and 60 µU was used in each assay. The pH optimum for amylase activity in the cell-free extract is between 8.5 and 9.0 (Figure 4.2). There is greater than 50 % of the optimal amylase activity between pH 6.0 and 9.8. Unlike the temperature profile there is no significant indication that there is more than one amylase activity present in the cell-free extract.

4.4.2 Effect of metal chelators on amylase activity

A phenomenon noticed during the early stages of developing a suitable purification protocol was the effect that EDTA had on the amylase activities. Originally, buffers used in the purification of the amylase activities contained EDTA as an inhibitor of divalent cation requiring proteases. When fractions containing amylase activity eluted from anion-exchange

were assayed in the presence and absence of EDTA a difference in activity was observed (Figure 4.3). Amylase II activity increased approximately 100 % in the absence of EDTA whereas amylase I activity decreased by approximately 20 %. Further observations were made using samples dialysed against 20 mM Tris-HCl (pH 8.5) and the addition of $CaCl_2$, NaCl or EGTA to the amylase assay. Both amylase activities decreased in the presence of EGTA (Figure 4.4). Amylase I activity was not significantly affected whereas amylase II activity decreased by 95 %. Amylase I showed no change in activity when assayed in 2 mM $CaCl_2$; amylase II however showed an approximately 4-fold increase in activity. This gives an overall increase in activity for amylase II from apoprotein to holoprotein of more than 70-fold.

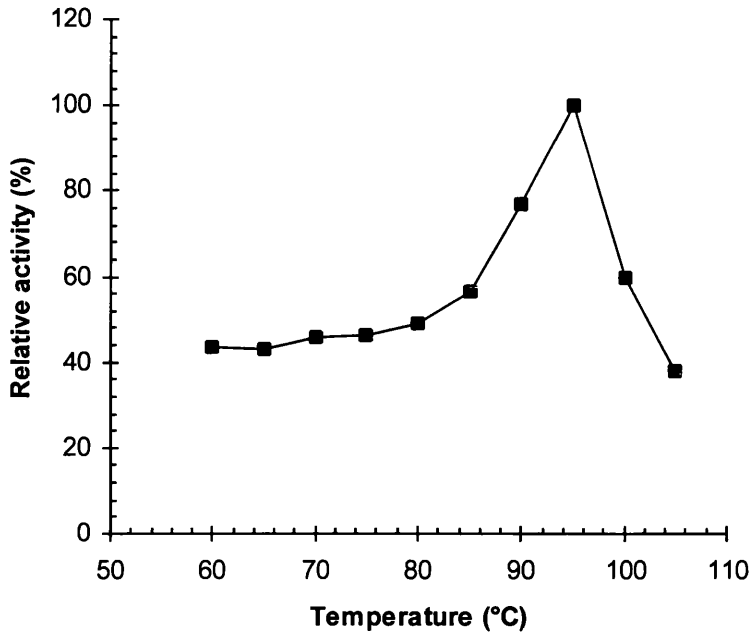


Figure 4.1 Temperature profile of amylase activity in cell-free extract of *T. natronophilum*

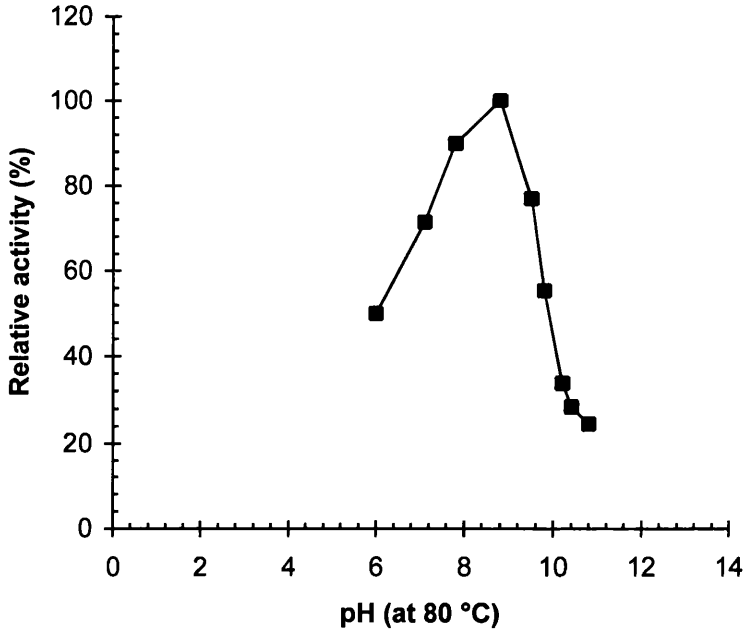


Figure 4.2 Normalised pH profile of amylase activity in cell-free extract of *T. natronophilum*

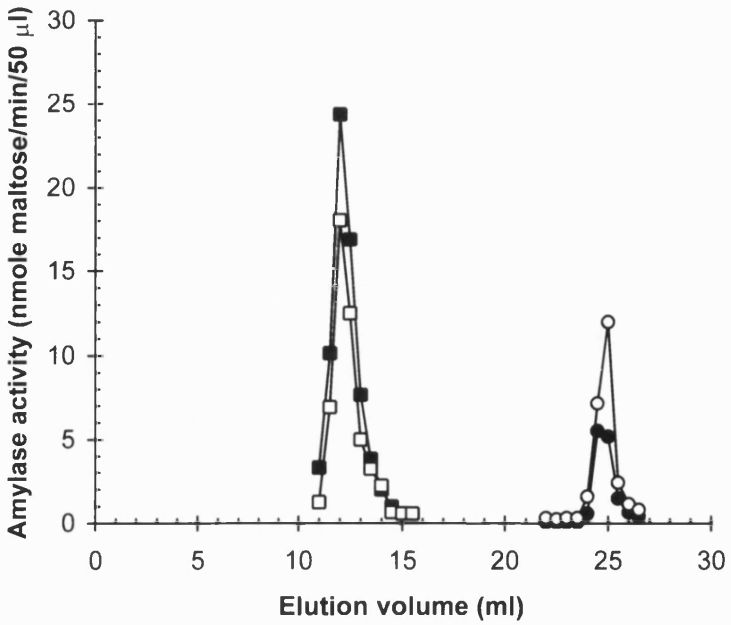


Figure 4.3 The effect of EDTA on amylase activities from *T. natronophilum*

Amylase I (■) and amylase II (●) activities were determined in the presence (solid) and absence of EDTA (hollow).

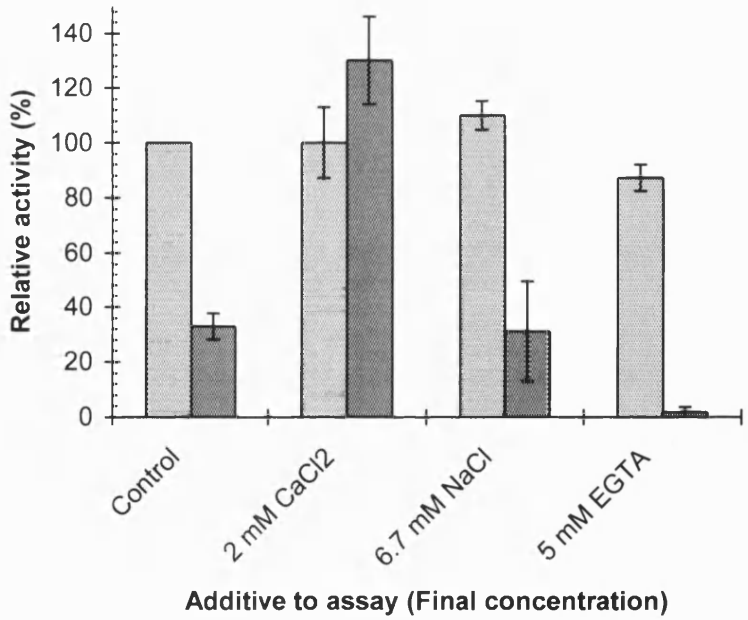


Figure 4.4 The effect of EGTA, $CaCl_2$ and NaCl on amylase activities from *T. natronophilum*

Amylase I activity is represented by light grey columns and amylase II activity is represented by dark grey columns. Error bars represent the upper and lower values, n=2.

4.4.3 Characterisation of the two purified amylases

4.4.3.1 Temperature optima

Aliquots of amylase I containing 8 μU of activity were assayed in triplicate at different temperatures between 65 and 100 °C. The temperature profile exhibited by amylase I shows a definite sharp peak where the activity can be seen to double with every 10 °C increase in temperature (Q_{10} rule) (Figure 4.5). The temperature range between which greater than 50 % of the optimal activity is seen spans 10 °C (88 - 98 °C). Under these assay conditions the temperature optimum was 95 °C.

Aliquots of amylase II containing 5 μU of activity were assayed in triplicate at different temperatures between 65 and 100 °C. The temperature profile differs from that of amylase I and does not show a defined peak of activity (Figure 4.6). Amylase II exhibits a temperature optimum of 80 °C and greater than 50 % of the optimal activity is observed at temperatures between 65 and 95 °C, a much broader range than seen with amylase I.

4.4.3.2 pH Optima

Aliquots of amylase I containing 1 μU of activity were assayed at different pH values in triplicate. Amylase I exhibits optimal activity at a pH of 10.2 and retains greater than 50 % of its optimal activity between pH 9.2 and 11.2 (Figure 4.7).

Aliquots of amylase II containing 4 μU of activity were assayed at different pH values in triplicate. Amylase II possesses a similar pH profile to that of amylase I with a slightly lower pH optimum of 9.6 (Figure 4.8). Greater than 50 % of the optimal amylase II activity is observed between pH 8.2 and 12.

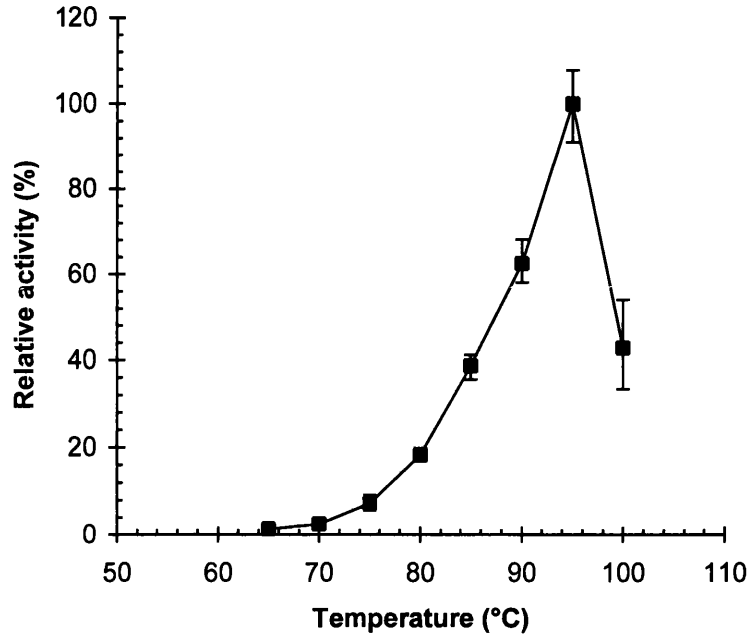


Figure 4.5 Temperature profile for amylase I from *T. natronophilum*
Error bars represent the upper and lower values, n=3.

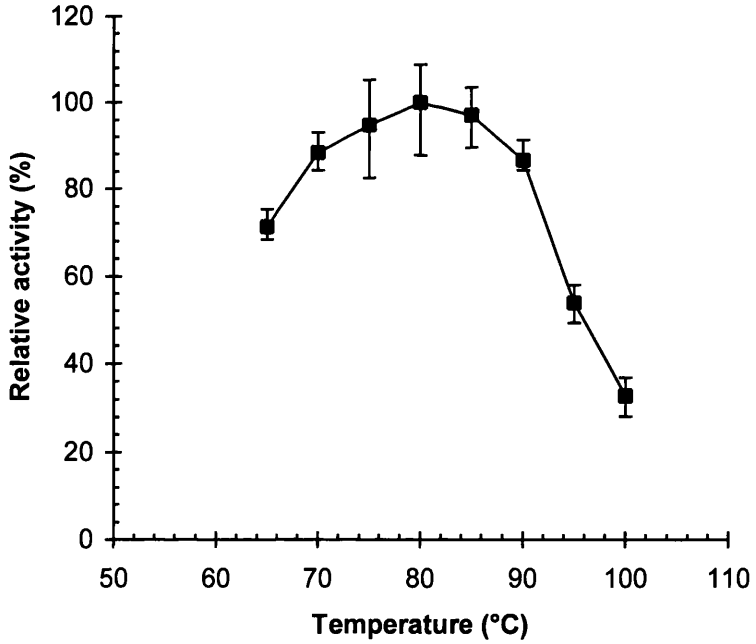


Figure 4.6 Temperature profile for amylase II from *T. natronophilum*
Error bars represent the upper and lower values, n=3.

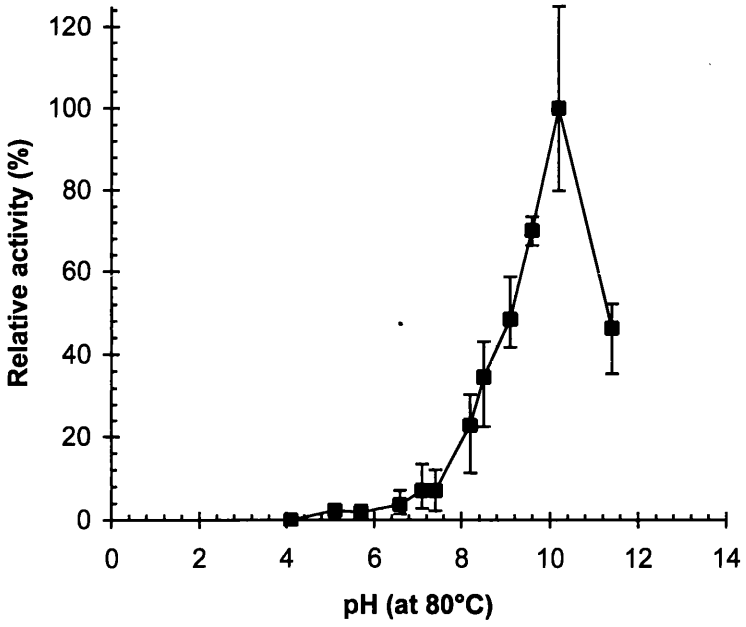


Figure 4.7 Normalised pH profile for amylase I from *T. natronophilum*
Error bars represent the upper and lower values, n=3.

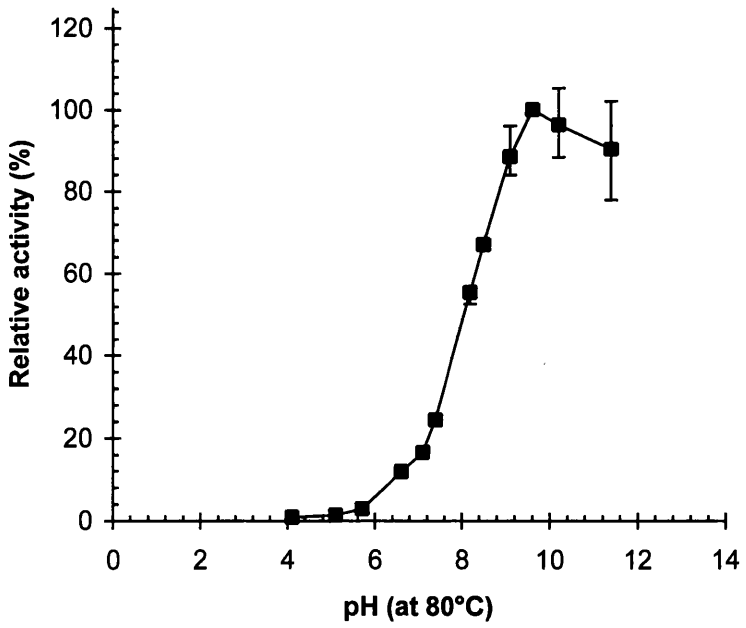


Figure 4.8 Normalised pH profile for amylase II from *T. natronophilum*
Error bars represent the upper and lower values, n=3.

4.4.3.3 HPLC analysis of hydrolysis products formed from starch

In order to determine the exact identities of the amylases it was essential to determine the products formed from starch. Products formed from starch were analysed by HPLC on a HPX 42A column (ion-exchange) which is capable of separating oligosaccharides containing 1 - 11 glucose units.

For comparison, two commercially available enzymes believed to have the same identity as amylases I and II were also studied. Tentative assignments based on Ca^{2+} requirement had been made and amylase I was thought to be a β-amylase while amylase II was believed to be an α-amylase. Novomyl® is a β-amylase produced by Novo Nordisk and is used as a "bread improving agent" in baking; Maxamyl® is an α-amylase produced by Gist-brocades and is used in the detergent industry.

Amylase I hydrolyses starch into a number of components ranging from maltose (dp2, dp - degree of polymerisation) to maltononaose (dp9) (Figure 4.9). During a 0 - 72 h incubation, it can be seen that hydrolysis products dp2 - dp9 increase in concentration up to 48 h, after which concentrations of dp3 - dp9 plateau. Interestingly, maltose continues to accumulate at a steady rate. In the 72 - 240 h incubation it can be seen that dp2 - dp9 increase in concentration, though the rate of production of dp2 is greater than the other products.

Novomyl® shows a similar pattern though more defined than amylase I (Figure 4.10). In a 0 - 32 h incubation relatively low amounts of glucose and dp3 - dp5 were produced compared to dp2, which was the major product.

Amylase II also hydrolyses starch into a variety of oligosaccharides (Figure 4.11). After 24 h of a 0 - 72 h incubation the major product is maltooctose (dp8), which is superseded at 32 h by maltotetraose (dp4), until this itself is surpassed by maltotriose (dp3) as the major product of the 72 h incubation. The other products show similar final concentrations except for glucose which is present in relatively low concentrations. After 72 h in the 72 - 240 h incubation, the rate of production of all products appear to plateau, with the exception of maltose (dp2) which continues to increase to become the major product.

Maxamyl® like amylase II produced a number of hydrolysis products from starch (Figure 4.12). After a 32 h incubation, maltopentaose (dp5) was the major product. The remaining oligosaccharides (glucose, dp2 - dp4, dp6 - dp11) were produced in varying amounts, with glucose and dp7 - dp11 being formed in the comparatively low amounts.

For direct comparisons between the products formed from starch by amylases I and II and Novomyl® and Maxamyl®, incubation times which reflect similar total hydrolysis of starch were chosen.

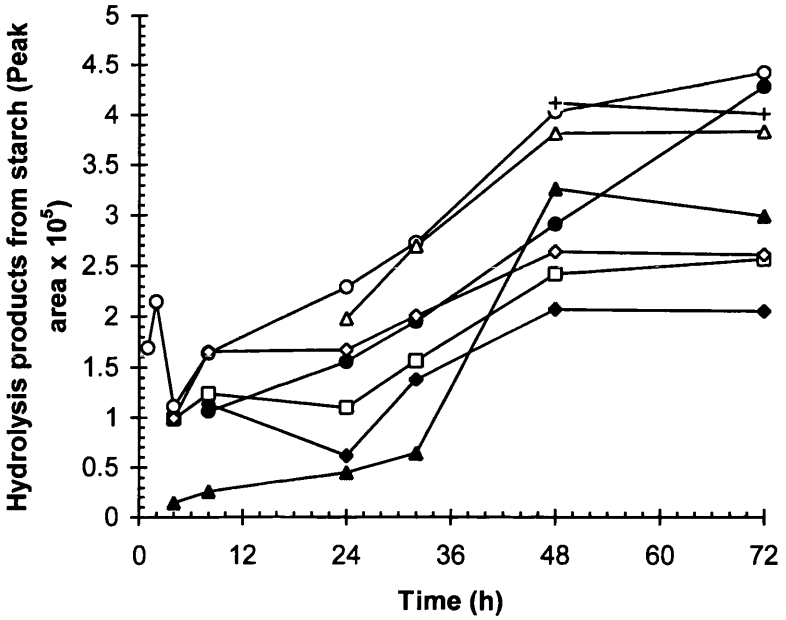
Amylase I and Novomyl® both formed maltose as their major hydrolysis product. However, amylase I produced a number of other oligosaccahrides in addition (Figure 4.13), while Novomyl® produced a fewer number of other oligosaccharides in lower amounts when compared with amylase I.

Amylase II and Maxamyl® formed different major products from starch (Figure 4.14). Amylase II produces maltose (dp2) in the greatest amount; dp3 - dp9 and glucose are produced in decreasing amounts. Maltopentaose (dp5) is produced as the major product when starch is hydrolysed by Maxamyl®.

Until this stage in the characterisation of the two amylases from *T. natronophilum*, it had been believed that amylase I was a β-amylase due to it's apparent lack of Ca^{2+} requirement and maltose being the major hydrolysis product from starch hydrolysis. However, preliminary studies using pullulan as a substrate showed that amylase I exhibited a remarkably greater activity against pullulan when compared with starch. In fact, amylase I was approximately 100 times more active on pullulan than starch. As this was discovered late during the HPLC analysis, which took part at Gist-brocades in The Netherlands, it was only possible to do a single incubation of amylase I with pullulan as substrate. Also it was not possible to perform a comparison due to a lack of a commercially available pullulanase.

Pullulan was hydrolysed 100 % after 65 h incubation with amylase I (Figure 4.15). The three major hydrolysis products were maltotriose (dp3), maltohexaose (dp6) and maltononaose (dp9). Other hydrolysis products (glucose, dp2, dp4, dp5 and dp7) were also produced in differing amounts.

(a)



(b)

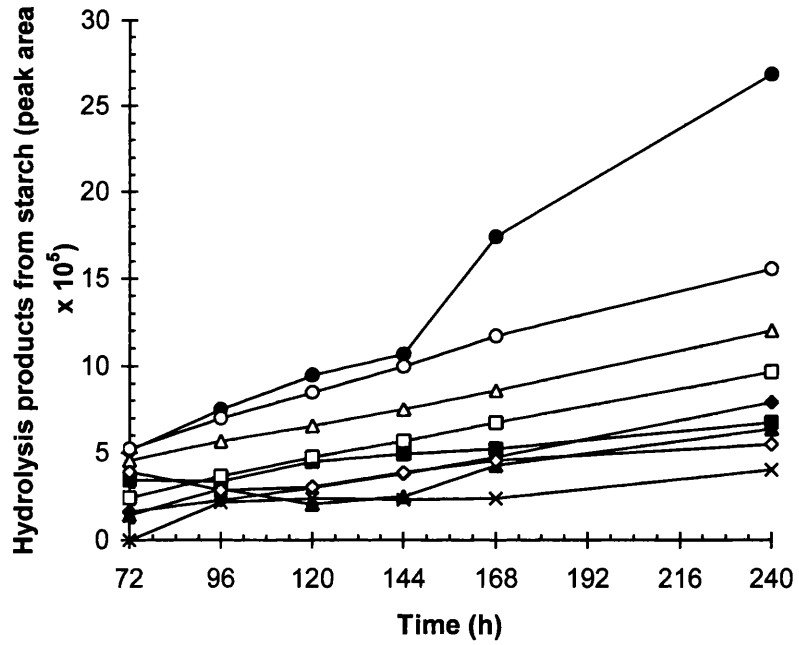


Figure 4.9 Hydrolysis products formed from starch by amylase I from *T. natronophilum*

(a) Represents 0 -72 h incubation, (b) represents 72-240 h incubation. The products are: Glucose (■), dp2-Maltose (●), dp3-Maltotriose (▲), dp4-Maltotetraose (◆), dp5-Maltopentaose (□), dp6-Maltohexaose (○), dp7-Maltoheptaose (△), dp8-Maltooctaose (◊), dp9-Maltononaose (+), dp10-Maltodecaose (x) and dp11-Maltoendecaose (✳)

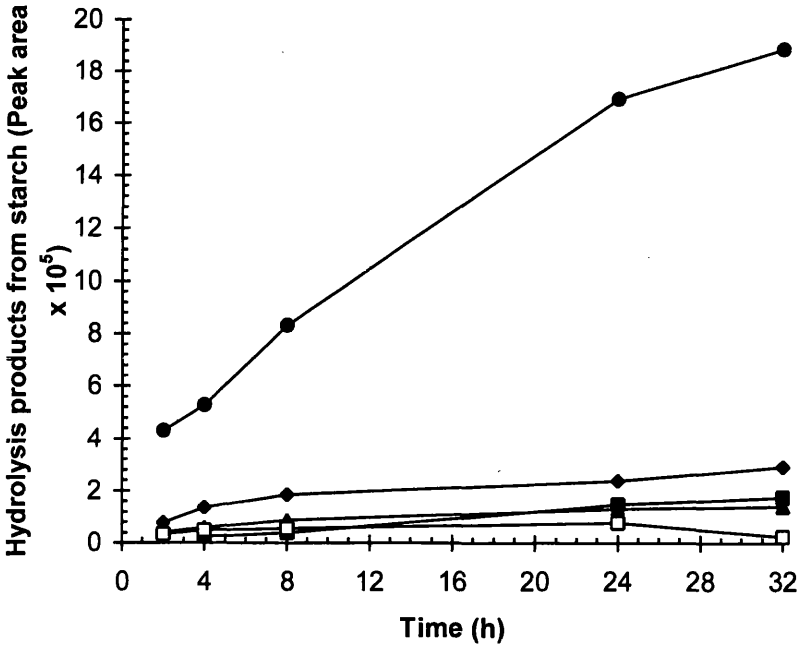
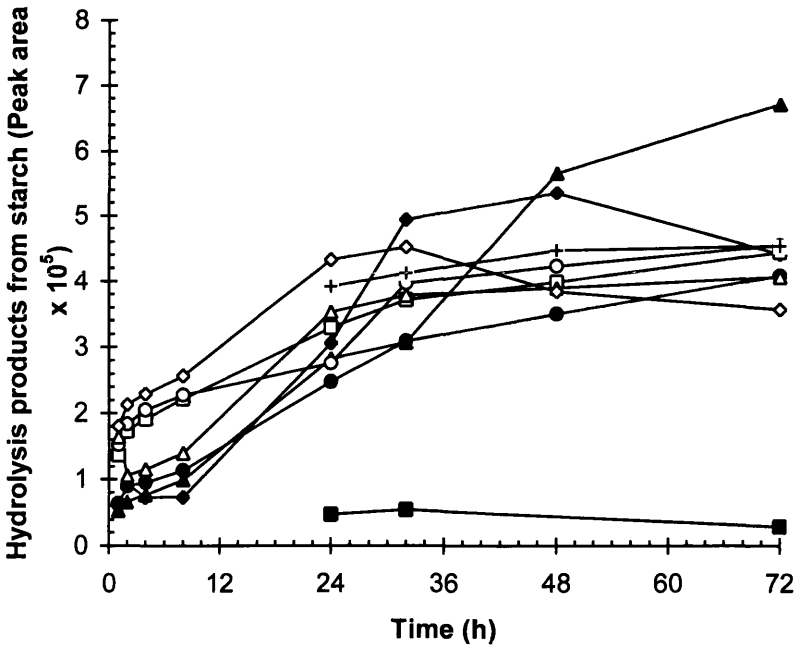


Figure 4.10 Hydrolysis products formed from starch by Novomyl®

The products are: Glucose (■), dp2-Maltose (●), dp3-Maltotriose (▲), dp4-Maltotetraose (◆), dp5-Maltopentaose (□), dp6-Maltohexaose (○), dp7-Maltoheptaose (△), dp8-Maltooctaose (◊), dp9-Maltononaose (+), dp10-Maltodecaose (x) and dp11-Maltoendecaose (✳)

(a)



(b)

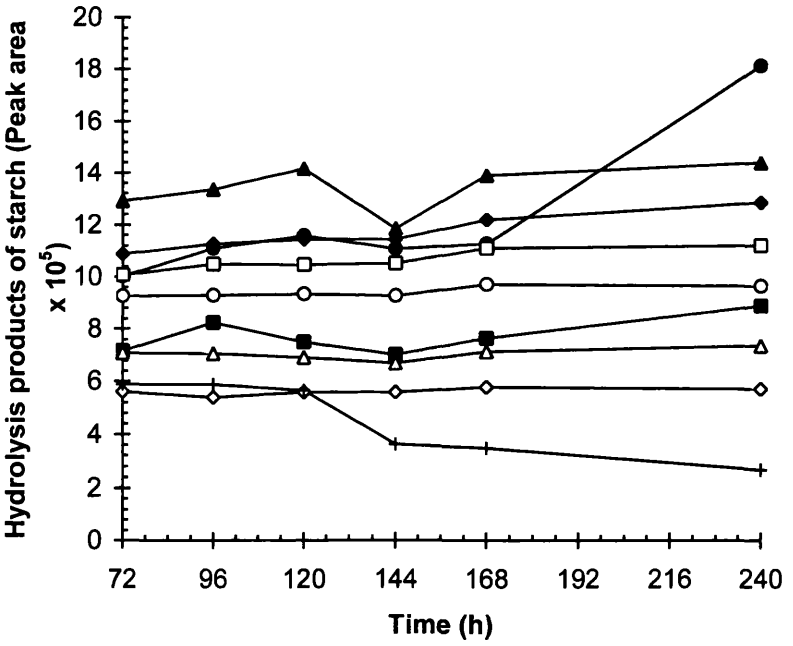


Figure 4.11 Hydrolysis products formed from starch by amylase II from *T. natronophilum*

(a) Represents 0 -72 h incubation, (b) represents 72-240 h incubation. The products are: Glucose (■), dp2-Maltose (●), dp3-Maltotriose (▲), dp4-Maltotetraose (◆), dp5-Maltopentaose (□), dp6-Maltohexaose (○), dp7-Maltoheptaose (△), dp8-Maltooctaose (◊), dp9-Maltononaose (+), dp10-Maltodecaose (x) and dp11-Maltoendecaose (✳)

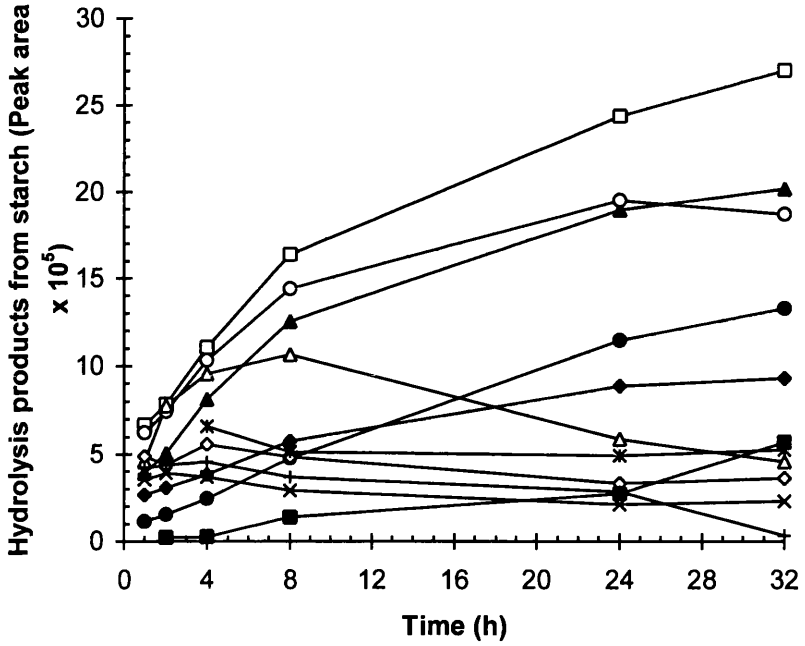
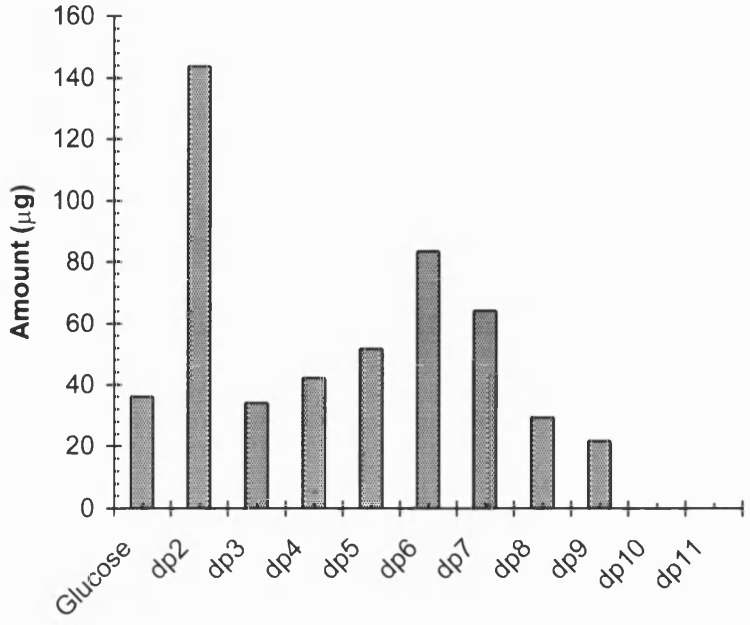


Figure 4.12 Hydrolysis products formed from starch by Maxamyl®

The products are: Glucose (■), dp2-Maltose (●), dp3-Maltotriose (▲), dp4-Maltotetraose (◆), dp5-Maltopentaose (□), dp6-Maltohexaose (○), dp7-Maltoheptaose (△), dp8-Maltooctaose (◊), dp9-Maltononaose (+), dp10-Maltodecaose (x) and dp11-Maltoendecaose (✳)

(a)



(b)

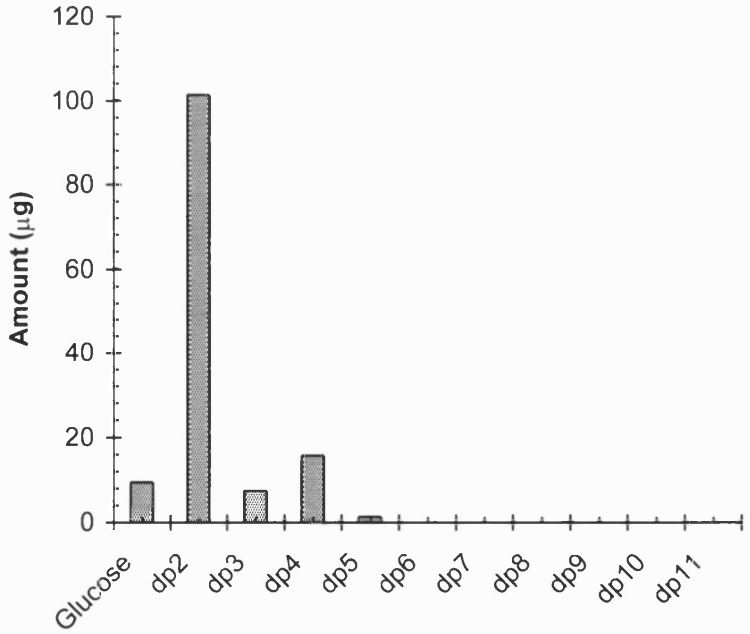


Figure 4.13 Amount of hydrolysis products formed from starch

(a) Shows the amount of hydrolysis products from starch after 240 h by amylase I. (b) Shows the amount of hydrolysis products from starch after 32 h by Novomyl®. Total starch hydrolysis was measured at 36 % and 40 % for amylase I and Novomyl®, respectively.

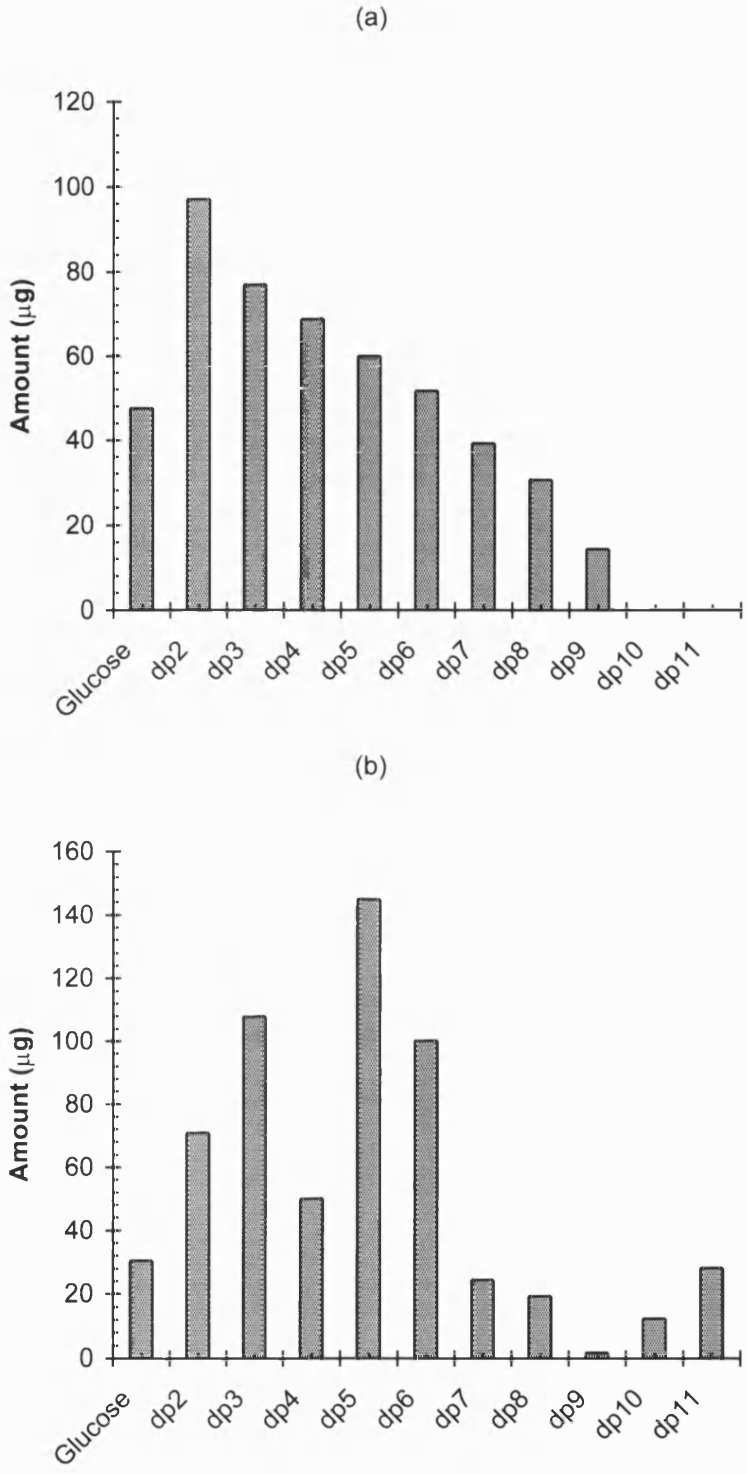


Figure 4.14 Amount of hydrolysis products formed from starch

(a) Shows the amount of hydrolysis products from starch after 240 h by amylase II. (b) Shows the amount of hydrolysis products from starch after 32 h by Maxamyl®. Total starch hydrolysis was measured at 71 % and 69 % for amylase II and Maxamyl®, respectively.

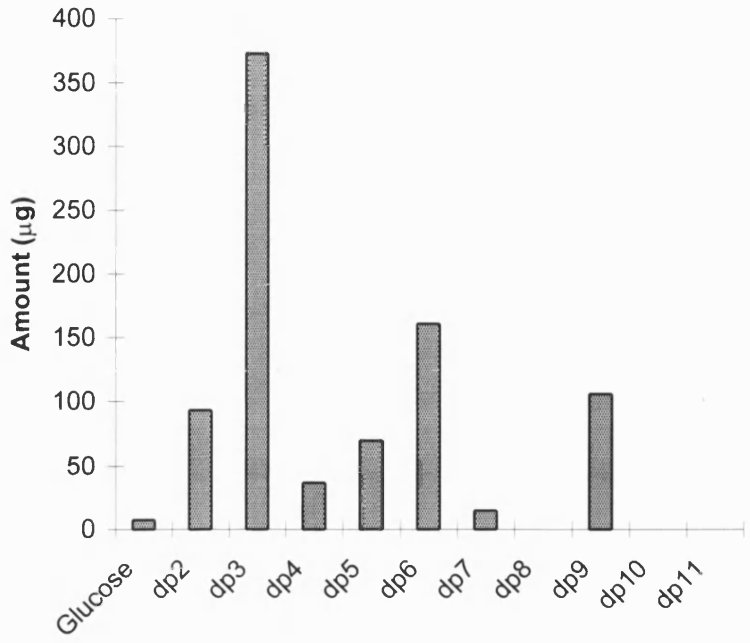


Figure 4.15 Amount of hydrolysis products formed from pullulan

Shows the amount of hydrolysis products from pullulan after 65 h by amylase I. Total pullulan hydrolysis was measured at 100 %.

4.4.3.4 Substrate specificity

Amylase I shows little activity against amylopectin and starch compared with pullulan (Figure 4.16). The activity on starch and one of it's components, amylopectin, is probably due to analogous regions within those structures to pullulan. No activity was observed against amylose, therefore, amylase I is not able to hydrolyse α-1,4-glycosidic bonds. Amylase I, thus, appears to be a pullulanase.

Amylase II unlike amylase I is unable to hydrolyse pullulan (Figure 4.17). Amylase II exhibits the highest activity against starch, though similar activities are seen with amylose and amylopectin, the two constituents of starch. Slight activity is also seen with the cyclodextrins, but they are negligible when compared to starch. Therefore amylase II seems to be an amylase.

4.4.3.5 Thermal inactivation studies

The pullulanase and amylase were incubated in the absence of substrates for up to 1 h and residual activity was measured using their respective assays. 200 μU/ml of pullulanase was incubated at 75, 90 and 95 °C and 100 μl aliquots were removed at varying time intervals and assayed for pullulanase activity. 100 μU/ml of amylase was incubated under similar conditions and 100 μl aliquots were assayed for amylase activity.

The pullulanase showed no decrease in activity when incubated at 75 °C (Figure 4.18). At 90 °C the pullulanase exhibited a half-life of approximately 84 min, which decreased to 17 min when the enzyme was incubated at 95 °C.

The amylase appeared to be less thermolabile when compared with the pullulanase. At 75 °C no decrease in amylase activity was observed after 60 min (Figure 4.19). At 90 °C a half-life of 602 min was observed, whilst at 95 °C the half-life of amylase activity was calculated to be 23 min.

As the enzymes were dialysed in the absence of Caclium ions or metal ion chelators it is not possible to infer any conclusions about thermal inactivation of the enzymes due to the effect of calcium ions.

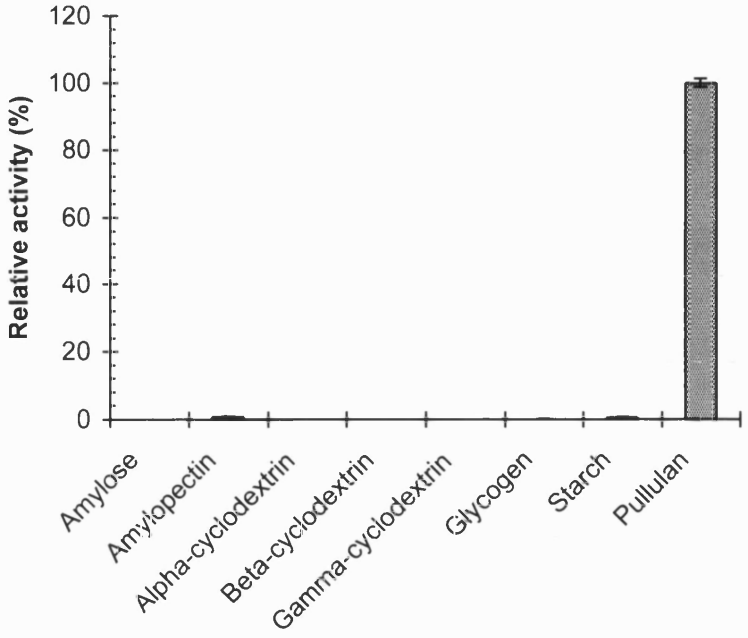


Figure 4.16 Substrate specificity of amylase I from *T. natronophilum*
Error bars represent the upper and lower values, n=4.

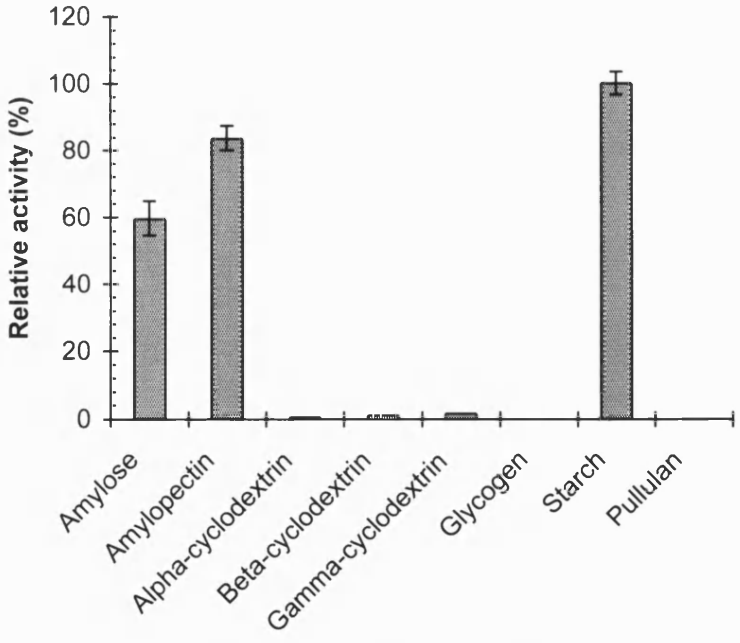


Figure 4.17 Substrate specificity of amylase II from *T. natronophilum*
Error bars represent the upper and lower values, n=4.

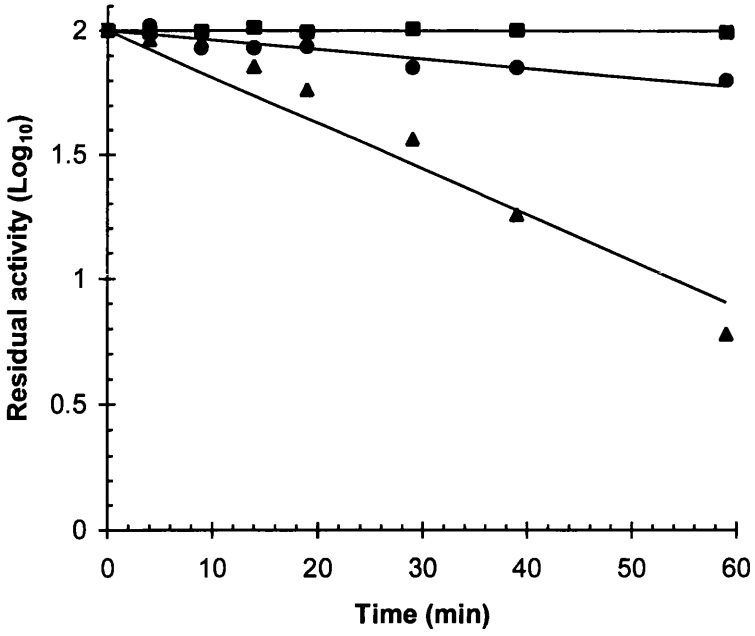


Figure 4.18 Thermal inactivation of pullulanase from *T. natronophilum* Incubation temperatures were 75 °C (■), 90 °C (●) and 95 °C (▲).

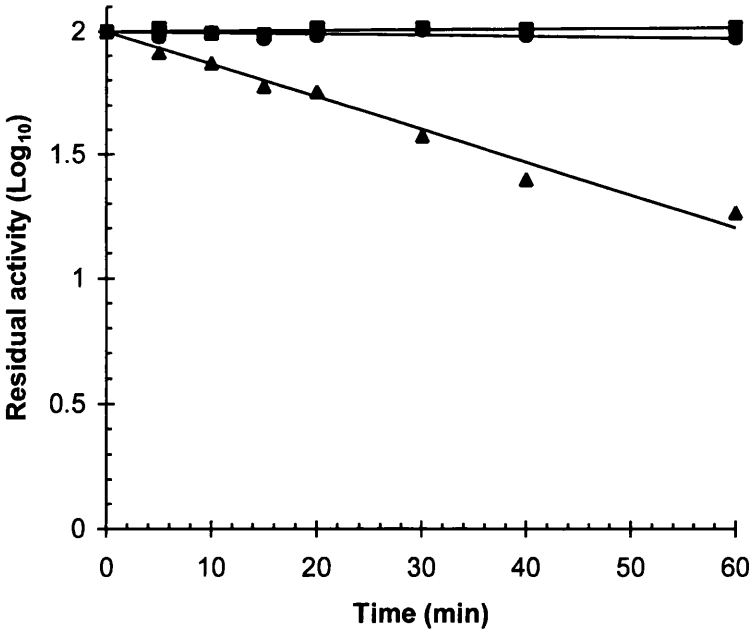


Figure 4.19 Thermal inactivation of amylase from *T. natronophilum* Incubation temperatures were 75 °C (■), 90 °C (●) and 95 °C (▲).

4.4.3.6 pH Optima

The pH optimum for the pullulanase was repeated using pullulan as the substrate to ascertain whether the optimum is the same as that seen when starch was used as the substrate. The pH optima were also determined for the amylase from *T. natronophilum*, pullulanase from *Klebsiella aerogenes* and Maxamyl®.

Aliquots containing 20 μU of pullulanase from *T. natronophilum*, 12 μU of pullulanase from *K. aerogenes* (55 °C, pH 5.5 (at 20 °C)), 7 μU of amylase from *T. natronophilum* and 32 μU of Maxamyl® (60 °C, pH 6.0 (at 20 °C)) were assayed at different pH values at 80, 55, 80 and 60 °C respectively.

The pullulanase from *T. natronophilum* was shown to have a different pH optimum in the presence of pullulan compared to that in the presence of starch (Figure 4.20). In the presence of pullulan, the pH optimum is approximately 7.5 and greater than 50 % of the optimal activity is observed between pH 3.9 and 8.9, a broader range than observed when assayed in the presence of starch.

The amylase from *T. natronophilum* showed similar results to those previously reported in this chapter (Figure 4.21). Optimum activity was observed at pH between 9.9 and 10.4 and over 50 % of the optimal activity was retained between pH 8.7 and 11.1.

The pullulanase from *K. aerogenes* and Maxamyl® both exhibited pH optima similar to those published (Figures 4.22 and 4.23). The pullulanase has a pH optimum of approximately 5.0. Maxamyl® exhibits a broad pH optimum range and appears to have optimal activity between pH 5 and 9.

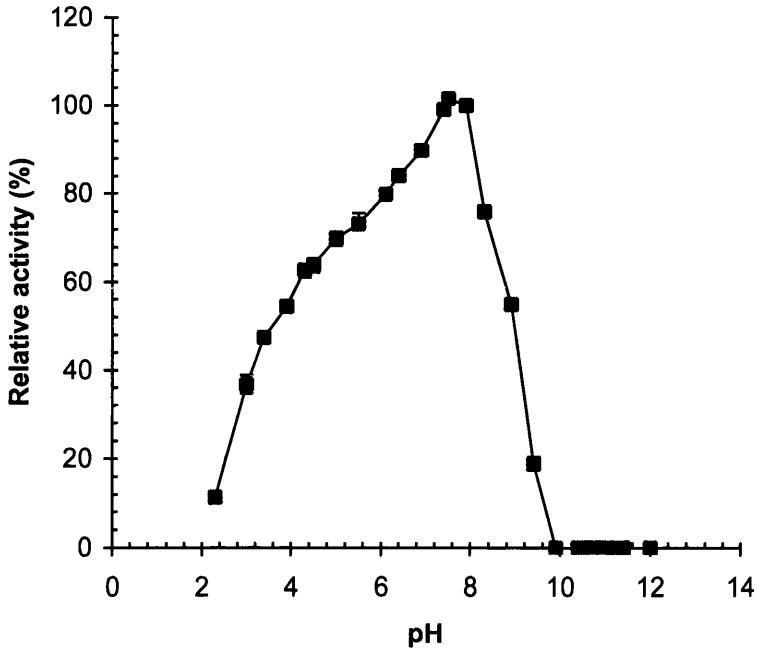


Figure 4.20 Normalised pH profile of pullulanase from *T. natronophilum*
Error bars represent the upper and lower values, n=5.

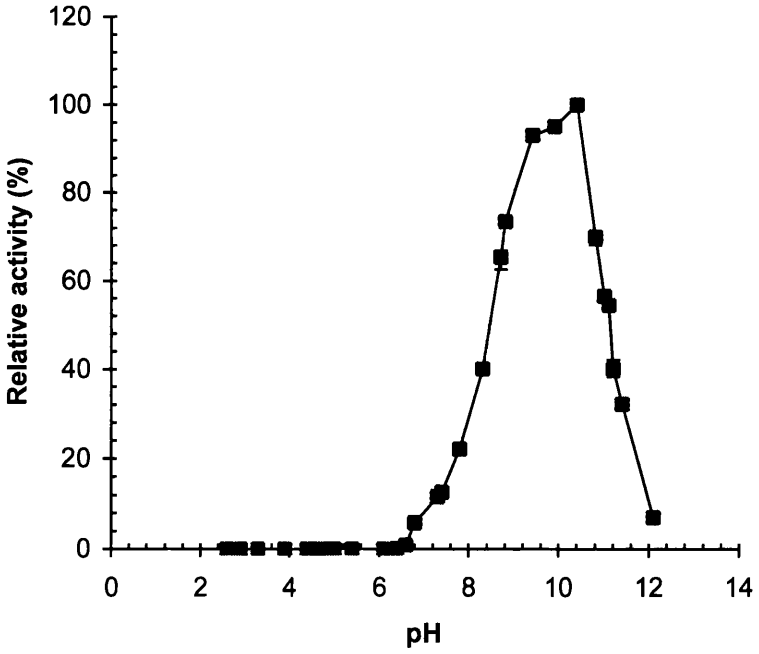


Figure 4.21 Normalised pH profile of amylase from *T. natronophilum*
Error bars represent the upper and lower values, n=5.

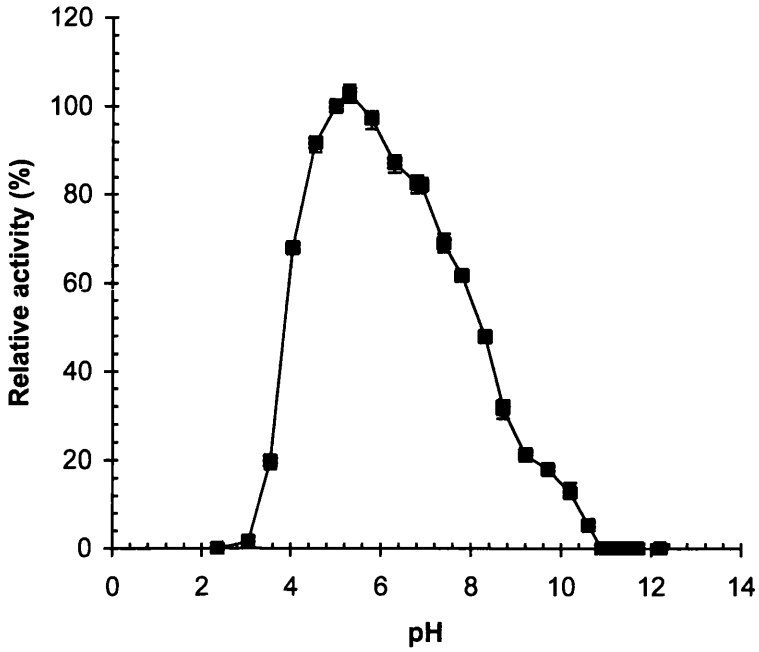


Figure 4.22 Normalised pH profile of pullulanase from *K. aerogenes*
Error bars represent the upper and lower values, n=5.

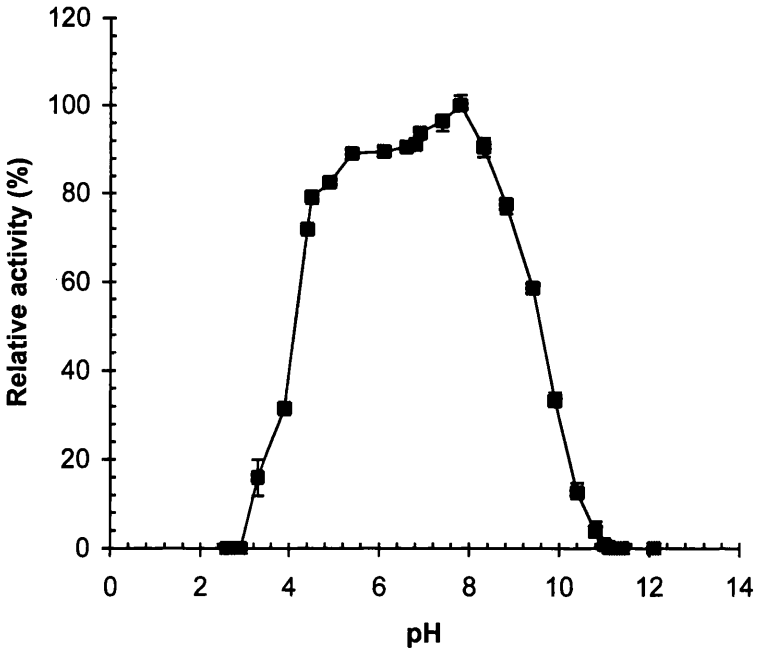


Figure 4.23 Normalised pH profile of Maxamyl®
Error bars represent the upper and lower values, n=5.

4.4.3.7 Temperature optimum of pullulanase

Aliquots containing 22 μU of pullulanase activity from *T. natronophilum* and aliquots containing 21 μU of pullulanase activity from *K. aerogenes* were assayed at different temperatures.

The pullulanase from *T. natronophilum* showed a slightly lower temperature optimum of 90 °C in the presence of pullulan compared to 95 °C in the presence of starch, previously determined (Figure 4.24). 50 % of total pullulanase activity is observed between 70 and 95 °C, spanning 25 °C instead of 10°C observed in the presence of starch.

The pullulanase from *K. aerogenes* exhibited at temperature optimum of 55 °C at pH 5.5 (at 20 °C) (Figure 4.25), a figure similar to that published.

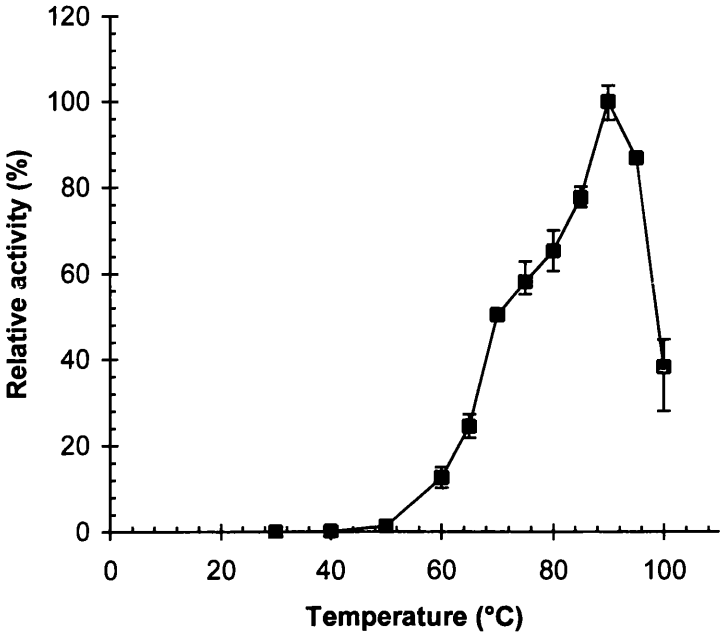


Figure 4.24 Temperature profile for pullulanase from *T. natronophilum*
Error bars represent the upper and lower values, n=4.

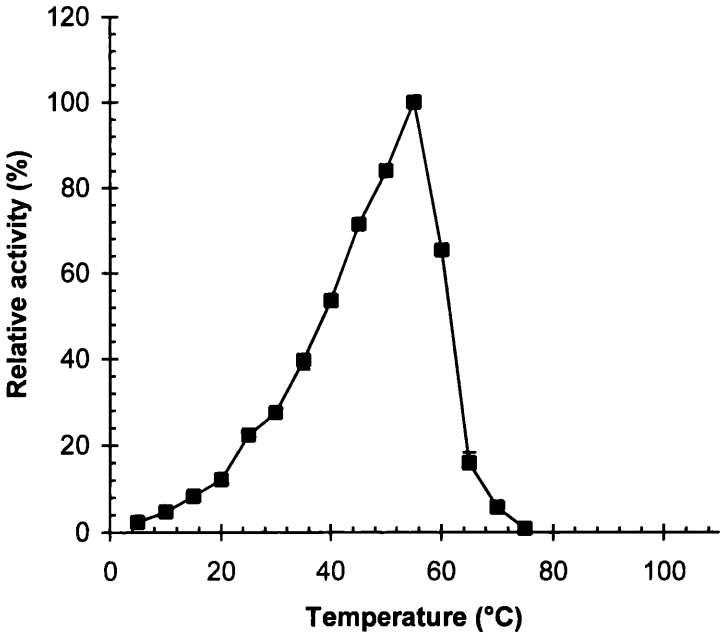


Figure 4.25 Temperature profile for pullulanase from *K. aerogenes*
Error bars represent the upper and lower values, n=4.

4.5 DISCUSSION

Temperature optimum studies performed on *T. natronophilum* cell-free extract revealed a peculiar profile. Peak activity was observed at 95 °C, but activity was still present at 60 °C and more confusing, activity appeared to remain constant between 60 and 80 °C. This uncharacteristic profile suggested that more than one amylase activity may be responsible for the profile. This was proved to be the case when two amylase activities were separated following anion-exchange chromatography (Chapter 3).

Unlike the temperature profile, the pH profile did not imply that there were numerous amylase activities present in the cell-free extract. The profile denoted that the various amylase activities seen in *T. natronophilum* exhibit a similar pH optimum of approximately pH 8.

A detrimental effect on one of the amylases activities due to the presence of EDTA was observed in the early stages of protein purification studies. EDTA chelates divalent metal cations and it was believed that amylase II required such metal ions for activity. Further studies using EGTA and $CaCl_2$ indicated that amylase II requires Ca^{2+} for activity. This observation has been reported for the majority of α-amylases [Janecek 1997]. Few exceptions are seen, *Pyrococcus furiosus* α-amylase being active and thermostable in the absence of Ca^{2+} [Koch *et al.* 1990, Laderman *et al.* 1993b]. *Pyrococcus woesei* α-amylase shows very slight stabilisation in the presence of Ca^{2+} and no loss of activity was observed in the presence of EDTA [Koch *et al.* 1991]. The most significant exception to the rule is an α-amylase from *Bacillus licheniformis* which appears to be inhibited by the presence of Ca^{2+} [Kumar *et al.* 1990].

β-amylases unlike α-amylases do not tend to require calcium ions for activity [Bernfeld 1955]. From this observation, coupled with the knowledge that a closely related organism, *Thermotoga maritima* possesses an α-amylase and a β-amylase [Schumann *et al.* 1991], amylase I and amylase II were tentatively classified as β-amylase and α-amylase, respectively.

The temperature optima profiles of amylase I and amylase II are very different in appearance. Amylase I has a temperature optimum of 95 °C and forms a much sharper peak than amylase II, which has a temperature optimum of 80 °C. If the temperature profiles for amylase I and amylase II were to be superimposed, the resulting profile would appear to be similar to the curious temperature profile observed for amylase activity in the cell-free extract of *T. natronophilum*.

Interestingly, the temperature optimum of amylase I of 95 °C is much higher than the optimal growth temperature of the organism; where at the upper temperature limit for growth there is only 20 % of the maximal activity. It is, however, within the temperature range found in the environment from which the organism was isolated (upto 95 °C). Amylase II possesses a much broader temperature profile, and the lower temperature optimum correlates better with the upper temperature limit for growth of the organism.

The temperature optima of amylase I and amylase II are above average when compared to amylases from *Bacillus* species [Morgan and Priest 1980, Krishnan and Chandra 1983, Bajpai and Bajpai 1987, Kochhar and Dua 1990, Ara *et al.* 1992, Jin *et al.* 1992, Joyet *et al.* 1992, Takase *et al.* 1992, Schwermann *et al.* 1994, Kim *et al.* 1995]. Amylases from 3 thermophilic fungi also possess similar temperature optima to the *T. natronophilum* enzymes [Obi and Odibo 1984, Jensen and Olsen 1992, Mellouli *et al.* 1996]. Amylases with higher temperature optima tend to belong not only to the thermophilic archaea, namely *Pyrococcus furiosus* [Koch *et al.* 1990, Laderman *et al.* 1993b], *Pyrococcus woesei* [Koch *et al.* 1991] and *Thermococcus profundus* [Chul-chung *et al.* 1995], but also to *Bacillus licheniformis* [Takasaki *et al.* 1993] and *Bacillus subtilis* [Pigott *et al.* 1984].

The pH profiles for amylase I and amylase II are very similar as are the pH optima for activity. Thermostable amylases isolated to date appear to be most active between pH 3 and 7 [Morgan and Priest 1980, Krishnan and Chandra 1983, Obi and Odibo 1984, Bajpai and Bajpai 1987, Koch *et al.* 1990, Kochhar and Dua 1990, Koch *et al.* 1991, Schumann *et al.* 1991, Jensen and Olsen 1992, Jin *et al.* 1992, Takase *et al.* 1992, Laderman *et al.* 1993b, Schwermann *et al.* 1994, Chul-chung *et al.* 1995]. Few bacterial amylases have pH optima at 9 - 12, and these tend to be produced by the *Bacillus* species [Ara *et al.* 1993, Kim *et al.* 1995, Mc Tigue *et al.* 1995]. However, they have temperature optima below 60 °C.

Amylase I hydrolysed starch into a number of oligosaccharides, the major product being maltose. Novomyl® also produced maltose in the greatest quantities from starch as well as a fewer number of other oligosaccharides compared to amylase I. These data seemed to uphold the theory that amylase I was a β-amylase. However, early substrate specificity studies indicated amylase I exhibited a considerably larger activity against pullulan when compared to starch. As time was limited, only one incubation was carried out on amylase I with pullulan as substrate. Maltotriose, maltohexaose and maltononaose were the major products formed in this incubation and this highlights that amylase I is able to hydrolyse pullulan into maltotriose from which pullulan is entirely composed. Pullulanases isolated from other organisms also produce maltotriose from hydrolysis of pullulan [Kim *et al.* 1993, Rüdiger *et al.* 1995, Koch *et al.* 1997]. The hydrolysis products seen from starch are probably due to the amylopectin portion, which contains α-1,6-glycosidic linkages in addition

to α-1,4 linkages. This would account for a final percentage hydrolysis of starch of 36 %, a value close to the percentage of starch that is composed of amylopectin.

Amylase II produces an array of hydrolysis products from starch, as does Maxamyl®. The major product formed by amylase II is maltose, other oligosaccharides dp3 - dp9 are produced in decreasing amounts and glucose is also present. This does not correlate with Maxamyl®, which has maltopentaose as the major hydrolysis product. However, in cases of excess hydrolysis, maltose and glucose are produced when more favourable hydrolysis substrates are not available. Studies into hydrolysis products from starch by α-amylases show that a proportion have maltose as the major product of hydrolysis and decreasing amounts from dp3+ [Candussio *et al.* 1990, Koch *et al.* 1990, Chul-chung *et al.* 1995, Dong *et al.* 1997]. Other α-amylases produce maltotriose [Schwermann *et al.* 1994], maltotetraose [Kim *et al.* 1995], maltopentaose [Stefanova and Emanuilova 1992], maltohexaose [Kim *et al.* 1992] and maltoheptaose [Koch *et al.* 1991] as major products from soluble starch. Therefore it would appear that these data support the belief that amylase II is an α-amylase.

Amylase I almost exclusively hydrolyses pullulan when compared with other α-1,4 and α-1,6-linked carbohydrates. Amylose is not hydrolysed by amylase I, whereas slight activity is seen against amylopectin and starch. The activity seen with starch is almost certainly due to the presence of amylopectin in starch. No activity was observed with α,β and γ-cyclodextrins and glycogen. Thus, amylase I does not appear to be able to hydrolyse α-1,4-glycosidic linkages and specifically hydrolyses the α-1,6-glycosidic linkages present in pullulan. The activity against amylopectin is probably due to regions within amylopectin that may mimic pullulan. Curiously, although glycogen is structurally similar to amylopectin, no activity was exhibited against this substrate. This may be due to the increased branching found within glycogen. From this study it seems likely that amylase I is a pullulanase (type-I).

Amylase II shows significant activity against starch and its components, amylose and amylopectin. No or slight activity was observed with the other substrates studied. Therefore, amylase II is able to hydrolyse α-1,4-glycosidic bonds, but not α-1,6-glycosidic bonds. A possible explanation for the greater activity observed with amylopectin compared to amylose may be due to the greater solubility of amylopectin than amylose. These data support the hypothesis that amylase II is an α-amylase.

In thermal inactivation studies, no loss of activity was observed when the amylase and pullulanase were incubated at 75 °C in the absence of substrate. At 90 °C the pullulanase

appears to less stable than the amylase and possesses a half-life some 7 times shorter than the amylase. At 95 °C both enzymes show similar half-lives of approximately 20 min. The discrepancy between the temperature optimum for the amylase and the stability of the enzyme at 90 °C may be due to the incubation in the presence of starch, though incubation in the presence of substrate tends to lead to elevated thermal stability [Chul-chung *et al.* 1995]. Due to the uncertainty about the presence of calcium ions, conclusions about the effects of calcium ions on thermal stability cannot be made.

Once the enzymes were established as an α-amylase and a pullulanase based on HPLC analysis and substrate specificity studies, pH optima were re-investigated. The α-amylase showed a similar profile to that elucidated in earlier studies. The pullulanase from *Klebsiella aerogenes* and Maxamyl® showed similar results to those published [Yamashita *et al.* 1994, Gist-brocades brochure]. Pullulanase from *T. natronophilum* exhibited a lower pH optimum when compared to activity in the presence of starch. The pH optimum of 7.5 is much closer to physiological pH than the pH optimum determined in the presence of starch. The elevation in pH optimum observed with starch may indicate that a higher pH is required to alter the enzyme in such a way that it is able to hydrolyse starch. The lower pH optimum observed with pullulan may have implications on the location of the pullulanase within *T. natronophilum.* A pH optimum of 7.5 implies that the pullulanase is likely to be intracellular rather than in direct contact with the alkaline environment. A possible role for an intracellular pullulanase may be as a debranching enzyme.

The temperature optimum of the pullulanase from *T. natronophilum* when incubated in the presence of pullulan was slightly lower (by -5 °C) than when incubated with starch. This would explain the discrepancy seen in thermal inactivation studies.

From a biotechnological perspective, both the α-amylase and pullulanase from *T. natronophilum* show promise. The most likely destination for an α-amylase and pullulanase such as these would be the detergent industry, though admittedly, the α-amylase would appear to be the better candidate of the two enzymes, due to it's high pH optimum. The α-amylase would not be suitable for the food industry. The α-amylase requires high pH for activity which would not lead to palatable products in the bakery or beverage industries. High Fructose Corn Syrup (HFCS) production processes require the absence of Ca^{2+} as it inhibits glucoamylase, which is used in subsequent steps. The pullulanase has a more moderate pH optimum and may be suitable as a debranching enzyme in the saccharyfication step of HFCS production. However, the temperature optimum of the pullulanase is greater than the glucoamylase used in the saccharyfication step, and therefore a more stable glucoamylase would also need to be introduced.

4.6 CONCLUSIONS

The two amylase activities isolated from *T. natronophilum* appear to be an α-amylase and pullulanase type-I based on HPLC analysis, substrate specificity studies and Ca^{2+} dependency.

The α-amylase has optimal activity at 80 °C and pH 10. The pullulanase has a slightly higher temperature optimum of 90 °C, but a much lower pH optimum of 7.5. This suggests that the pullulanase may be intracellular and that the α-amylase is associated with the "toga" outside the pH control of the cell.

From the studies carried out so far, it would appear that the α-amylase and pullulanase would be suited to the detergent industry.

CHAPTER 5

Partial amino-acid sequencing of α-amylase and pullulanase from *Thermopallium natronophilum*

5.1 INTRODUCTION

In order to locate and isolate the genes responsible for the α-amylase and pullulanase it was necessary to determine partial protein sequence, which would provide the information for a cloning strategy. This chapter deals with the determination of such protein sequences and there subsequent computer-based analysis using other protein and nucleotide sequences currently available.

5.2 MATERIALS

The transfer of proteins from polyacrylamide gels to PVDF membranes (Immobilon P, Millipore, Watford, UK) was achieved using a Novablot apparatus (Pharmacia, Uppsala, Sweden).

Computer-based sequence analysis was performed using the GCG package (Genetics Computer Group, Inc., Madison, WI, USA)

Other materials and their suppliers mentioned in this chapter are listed in chapter 2.

5.3 METHODS

5.3.1 Transfer of proteins from polyacrylamide gels to PVDF membranes

The protein samples were electrophoresed through a 10 % (w/v) polyacrylamide gel which, without staining, was prepared for semi-dry protein blotting by incubating in transfer buffer (50 mM Tris, 50 mM glycine, 20 % (v/v) methanol) for 15 min. The PVDF membrane, cut to the same size as the gel, was washed in methanol for 3 s, water for 2 min and transfer buffer for 3 min. Fourteen oblongs of Whatman 3MM chromatography paper, cut to the same size as the gel, were submerged in transfer buffer. The electroblot was assembled in a Novablot apparatus in the following order: positive electrode, 7 filters, 2 PVDF

membranes, gel, 7 filters and negative electrode. Transfer of the protein bands to the membrane was achieved by applying 1 mA/cm^2 for 2 - 3 h. The membrane was then stained with 0.2 % (w/v) Coomassie Brilliant Blue R in 20 % (v/v) methanol, 0.5 % (v/v) acetic acid for 20 min at room temperature, and destained using 30 % (v/v) methanol at room temperature until the protein bands became visible. Protein bands that required amino acid sequencing were cut out and air dried. Sequencing was achieved using the method of Edman degradation at Eurosequence (Eurosequence bv., Groningen, The Netherlands).

5.3.2 Internal sequence analysis

Internal sequence analysis was achieved by *in situ* tryptic digestion of the protein band in an SDS-PAGE gel, followed by extraction, RP-HPLC purification, selection and sequence analysis of suitable fragments. This technique was carried out externally by Eurosequence.

5.4 RESULTS

5.4.1 Determination of amino acid sequence

Two identical SDS polyacrylamide gels containing 25 μg of pullulanase and 65 μg of α-amylase each were electrophoresed. After electrophoresis one gel was electroblotted onto a nitrocellulose membrane, the protein was visualised by staining with Coomassie and the membrane was air-dried before the relevant bands were excised. The second SDS polyacrylamide gel was stained with Coomassie and the relevant bands were excised. Both the blotted and *In situ* protein bands were sent to Eurosequence for N-terminal and internal amino acid sequence determination, respectively.

Unfortunately, N-terminal sequencing did not yield any results possibly due to blocked N-termini. Internal sequencing gave relatively large protein sequences for the pullulanase and α-amylase (Figure 5.1).

Pullulanase	Y I G D G A W E A V L E G D **D** E **G** X F Y **R**
α-Amylase	I G L P S V M T E P W N P I G G S N W I F D M M L I R

Figure 5.1 Internal amino acid sequence of *T. natronophilum* hydrolases
Tentative residues are typed in bold characters and X represents unknown amino acids.

5.5 DISCUSSION

5.5.1 Primary structure

The amino acid sequences for the pullulanase and α-amylase from *Thermopallium natronophilum* were analysed using various programs available in the GCG package. The sequences were used to find matches within Swissprot, PIR and GenEMBL databases using Blast, FastA and TfastA. Blast(p) (Basic Local Alignment Search Tool) uses the method of Altschul *et al.* [1990]. FastA and TfastA use the method of Pearson and Lipman [1988].

5.5.1.1 Pullulanase

When the pullulanase amino acid sequence was used to search Swissprot using the Blast program, the majority of hits were with proteins involved with the synthesis of DNA and RNA. However, a match with a 1,4-α-glucan branching enzyme (EC 2.4.1.18) (glycogen branching enzyme) from *Streptomyces aureofaciens* was made (Table 5.1). This match was also observed when the PIR database was searched using the Blast program [Homerova and Kormanec 1994]. The FastA search provided much more promising results compared to the Blast searches. Using the Swissprot database, the FastA program matched the pullulanase sequence to endo-1,4-β-xylanase b (EC 3.2.1.8) [Lin and Thomson 1991] from *Butyrivibrio fibrisolvens*. More interestingly, the FastA program also matched the pullulanase to the pullulanases from *Klebsiella pneumoniae* and *Klebsiella aerogenes* [Kornacker and Pugsley 1990, Katsuragi *et al.* 1987]. A search carried out with Blastp using the National Center for Biotechnology Information (NCBI, Washington DC, WA, USA, URL: http://www.ncbi.nlm.nih.gov/) matched the pullulanase with the pullulanase from *Thermotoga maritima*, with 65 % identity for amino acids from 1 - 20 inclusive. This was the greatest identity match which reflects the close relatedness between *T. natronophilum* and *Thermotoga maritima*. This would support the belief that there is a pullulanase type-I present in *Thermopallium natronophilum*. The TfastA program failed to find anything of interest and predominantly returned sequences for hypothetical proteins from the ongoing genome sequencing projects.

5.5.1.2 α-Amylase

When the α-amylase amino acid sequence was used to search Swissprot using the Blast program, a match was obtained with glucan endo-1,3-β-glucosidase (EC 3.2.1.39) from *Pisum sativum* (garden pea) [Chang *et al.* 1993] (Table 5.2). Analysis using Blast and searching the PIR database also hit upon glucan endo-1,3-glucosidase from *Pisum sativum* [Chang *et al.* 1992] and from *Saccharomyces cerevisiae* [Aljinovic *et al.* 1994 (Accession number only - s45914.pr2)]; also highlighted was levansucrase (EC 2.4.1.10) from

Zymomonas mobilis [Song *et al.* 1993, Kyono *et al.* 1995]. All these enzymes belong to the α-amylase family (Table 1.3) and share similar secondary/tertiary structures, namely an $(\alpha/\beta)_8$-barrel. Although α-amylase from *T. natronophilum* did not match-up with α-amylases from other species, this is not unexpected as there can be less than 10% identity between α-amylases from different species [Nakajima *et al.* 1986]. The FastA program did not return any sequences of interest and TfastA returned similar results to those for pullulanase. No significant matches were returned when Blastp was executed using the resources at NCBI.

Enzyme	Sequence	Identity
Pullulanase[a]	1YIGDGAWEAVLEGDDEGXFYR21	
1,4-α-Glucan branching enzyme	207LGASGVWELFLPGVAEGALYK227	47.1
Endo-1,4-β-xylanase b	139PGIIYAWDVVNEIVDEGAFRK159	57.1
Pullulanase[b]	345DSASGAWSWQGGSDLKGAFYR365	42.1
Pullulanase[c]	345DSASGAWSWQGGSDLKGAFYR365	42.1
Pullulanase[d]	263YKGNGVWEAVVEGDLDGVFYL287	65.0

Table 5.1 Sequence similarity search results for pullulanase from *T. natronophilum*
Bold letters represent the sequence returned by the search program. Residues highlighted in red denote exact matches and those highlighted in yellow represent similar residues. For the duplicated enzymes the superscript letters represent, a, *T. natronophilum*, b, *K. pneumoniae*, c, *K. aerogenes* and d, *T. maritima*.

Enzyme	Sequence	Identity
α-Amylase	1IGLPSVMTEPWNPIGGSNWIFDMMLIR27	
Glucan endo-1,3-β-glucosidase[a]	118KIKYIAVGNEVSPVGGSSWLAQYVLPA144	28.6
Glucan endo-1,3-β-glucosidase[b]	262QGLPVIISDGNWPQQWADWVKEKHFSE288	31.6
Levansucrase	82NRNDGARIGYFYSRGGSNWIFGGHLLK108	61.5

Table 5.2 Sequence similarity search results for α-amylase from *T. natronophilum*
Bold letters represent the sequence returned by the search program. Residues highlighted in red denote exact matches and those highlighted in yellow represent similar residues. For the duplicated enzymes the superscript letters represent, a, *P. sativum* and b, *S. cerevisiae*.

5.5.2 Secondary structure

As a measure of the accuracy of the matches made at primary structure level, secondary structure analysis was carried out on the matching sequence segments. As a general rule you would expect the same enzymes from different organisms are expected to possess similar secondary structures.

Secondary structure analysis was achieved using a prediction program, PEPTIDESTRUCTURE in the GCG package. The secondary structure was predicted by the method of Chou and Fasman [1978].

5.5.2.1 Pullulanase

The predicted secondary structure of the pullulanase from *Thermopallium natronophilum* shows an α-helix flanked by turns (Figure 5.2). A similar predicted composition was elucidated for the pullulanase from *T. maritima*, but not for the pullulanases from *Klebsiella sp.* This may be an indication of the close relatedness between *T. natronophilum* and *T. maritima*, whereas *K. pneumoniae and K. aerogenes* are less-related to either organism. It must however be emphasised that these predicted secondary structures are small segments of the overall proteins and may differ from the actual structures as determined by x-ray crystallography.

5.5.2.2 α-Amylase

The α-amylase consists of a predicted turn follower immediately by a helix. This composition is not seen in either of the glucan endo-1,3-β-glucosidases or levansucrase. Whether this is due to the enzymes originating from a different branch of the phylogenetic tree or incorrect matches is difficult to resolve.

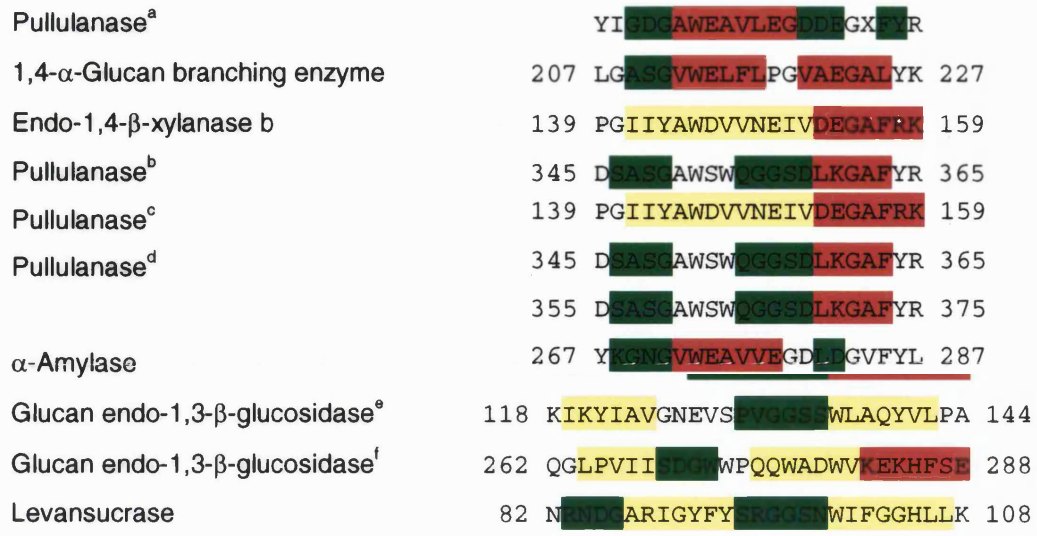


Figure 5.2 Secondary structure prediction

Secondary structure was predicted using PEPTIDESTRUCTURE program in GCG and via the method of Chou and Fasman [1978]. Residues highlighted in green denote a turn, residues in red represent helix and yellow depicts sheet. For the duplicated enzymes the superscript letters represent, a, *T. natronophilum*, b, *K. pneumoniae*, c, *K. aerogenes*, d, *T. maritima*, e, *P. sativum* and f, *S. cerevisiae*.

5.6 CONCLUSIONS

It is more than likely that the pullulanase from *T. natronophilum* is indeed a pullulanase type-I, given the data accumulated so far. It shows remarkable identity with the *T. maritima* enzyme and also shares a similar predicted secondary structure. The α-amylase unfortunately did not provide similar results, but did show some sequence identity with members of the α-amylase family. All of the data collected so far for the α-amylase would support its classification, and this discrepancy in database searching may be due to the fact that α-amylases can have less than 10 % sequence identity between species.

CHAPTER 6

Creation and screening of a *Thermopallium natronophilum* genomic DNA library in λ bacteriophage

6.1 INTRODUCTION

Once protein sequence had been determined for pullulanase and α-amylase from *T. natronophilum*, the next logical step was to isolate the genes responsible. This and the following chapters outline work undertaken to attempt to achieve this goal. Before attempting to locate the genes responsible it was necessary to create a gDNA library for *T. natronophilum* for easier manipulation.

6.2 MATERIALS

The Lambda cloning kit, Gigapack® III Gold Packaging Extract, was purchased from Strategene (La Jolla CA, USA).

Other materials and their suppliers mentioned in this chapter are listed in chapter 2.

6.3 METHODS

6.3.1 Creation of λ library

6.3.1.1 Pilot digestion

To 180 μg of gDNA in a volume of 180 μl was added 20 μl of *Sau* 3AI reaction buffer (10x) in a 1.6 ml microcentrifuge tube. The contents were heated to 70 °C for 10 min to unravel the gDNA. 40 μl of this mixture was transferred to a sterile 0.6 ml microcentrifuge tube labelled 1, and the remaining mixture was further aliquoted (20 μl) into sterile 0.6 ml microcentrifuge tubes labelled 2 - 9; all the tubes were stored on ice. 10 U of the restriction endonuclease *Sau* 3AI was added to tube 1 and the contents were carefully mixed. 20 μl of the mixture contained in tube 1 was transferred to tube 2 and the contents were carefully mixed. This

procedure was carried out for the remaining tubes up to tube 9. The tubes were incubated at 37 °C for 30 min, after which the reactions were stopped by the addition of 0.5 M EDTA (pH 8.0) to a final concentration of 10 mM. 10 µl of each tube was then analysed by electrophoresis on a 0.5 % (w/v) agarose gel.

6.3.1.2 Scale-up digestion

3 x 180 µl aliquots of gDNA were mixed with 20 µl of *Sau* 3AI reaction buffer (10x) in 1.6 ml microcentrifuge tubes. The mixtures were then heated to 70 °C for 10 min to unravel the gDNA. Differing amounts of *Sau* 3AI was added to each tube, 0.7 U, 0.35 U and 0.175 U, and their contents were carefully mixed. The tubes were incubated at 37 °C for 30 min, after which the reactions were stopped with the addition of 0.5 M EDTA (pH 8.0) to a final concentration of 10 mM. 10 µl of each reaction mixture was analysed on a 0.5 % (w/v) agarose gel.

6.3.1.3 Size fractionation by centrifugation through a salt gradient

The remaining mixtures from the scaled-up digestion were combined and extracted once with phenol:chloroform:isoamyl alcohol (25:24:1(v:v:v)). The aqueous layer was recovered after centrifugation in a microcentrifuge at 12,000 x *g* for 5 min and was then mixed with 1/10 volume of 3 M potassium acetate (pH 5.2). Nucleic acids were precipitated by the addition of 2 volumes of ice cold ethanol and pelleted by centrifugation at 12,000 x *g* for 10 min at 4 °C. The pellet was washed with a small volume of ice cold 70 % (v/v) ethanol and repelleted by centrifugation at 12,000 x *g* for 10 min at 4 °C. The pellet was then redissolved in 500 µl of sterile deionised water. Recovery was verified by analysing 10 µl of the sample on a 0.5 % (w/v) agarose gel.

Disposable centrifuge tubes (30 ml) containing salt gradients were made by carefully layering 5 ml of decreasing concentrations of NaCl (25, 20, 15, 10, 5 % (w/v)), so that interfaces between different concentrations were clearly visible. The salt gradients were allowed to diffuse to create a continuous gradient by incubating at 4 °C for 3 - 4 h. Immediately prior to centrifugation, the 500 µl sample was carefully pipetted onto the top of the salt gradient. Separation of nucleic acid fragments was achieved by centrifugation using a swing-out rotor (Sorvall, AH-629) at 68,400 x *g* at 15 °C for 16 h. After centrifugation, a sterile needle was used to perforate the bottom of the centrifuge tube and 600 - 700 µl fractions were collected. 5 µl of each fraction was mixed with 11.7 µl of sterile deionised water and 3.3 µl of 6x loading dye and analysed on a 0.5 % (w/v) agarose gel.

Tubes containing fragments sized between 15 - 18 kb were pooled and an equal volume of sterile deionised water was added prior to nucleic acid precipitation by the addition of

2 volumes of ice cold ethanol. The nucleic acids were pelleted by centrifugation at 12,000 x *g* for 10 min at 4 °C. The pellet was washed and repelleted by centrifugation at 12,000 x *g* for 10 min at 4 °C, prior to dissolving in 50 µl of sterile deionised water. 5 µl of sample was analysed on a 0.5 % (w/v) agarose gel.

6.3.1.4 Ligation with λ vector and packaging into bacteriophage

0.3 µg of gDNA fragments was ligated into EMBL3 arms using T4 DNA ligase (2 Weiss units) in a total volume of 5 µl and incubated at 15 °C overnight.

Following ligation, the ligated mixture was placed on ice. 4 µl of the ligated mixture was added to a tube containing packaging extract (25 µl) which had been quickly thawed by holding between fingers. The resulting solution was carefully mixed using a pipette tip, whilst being careful not to introduce air bubbles. The tube was centrifuged at 12,000 x *g* for 5 s to ensure the contents were at the bottom of the tube. The mixture was incubated at 22 °C for 2 h, after which it was quenched by the addition of 500 µl of SM buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgSO_4$, 0.01 % (w/v) gelatin). 20 µl of chloroform was added to the tube and its contents were gently mixed; debris was pelleted by centrifugation at 12,000 x *g* for 10 s. The supernatant was recovered and stored over a drop of chloroform at 4 °C.

6.3.2 5' End labelling of oligonucleotides with [γ^{32}P] dATP

A 20 pmole quantity of oligonucleotide was incubated with 70 µC$_i$ [γ^{32}P] dATP and 14 U T4 polynucleotide kinase in a 40 µl volume of 1x kinase buffer (supplied by the manufacturer) at 37 °C for 30 min.

Other procedures mentioned in this chapter are outlined in chapter 2.

6.4 RESULTS

6.4.1 Creation of *T. natronophilum* gDNA library in λ bacteriophage

Although the gDNA preparation was slightly degraded (Figure 6.1a), this would not have a deleterious effect on the library preparation as only fragments of gDNA with *Sau* 3AI cut ends would be included. From the pilot digestion of gDNA, *Sau* 3AI concentrations of 0.078 and 0.040 U in a 20 μl reaction volume were selected for the scale-up digestion as these concentrations would appear to give rise to the highest proportions of gDNA fragments of 15 kb (Figure 6.1b). Also to gain more 15 kb fragments a third digestion was carried out with 0.020 U of *Sau* 3AI. The scale-up digestion was checked prior to pooling and purification (Figure 6.1c and d).

Following purification, the sample was size fractionated by centrifugation through a salt gradient. A small sample of each fraction was analysed by electrophoresis (Figure 6.1e) and fractions from 2 to 7 (inclusive) were pooled and concentrated by precipitation with ethanol and resuspended in 50 μl of sterile de-ionised water, of which a sample was analysed by electrophoresis (Figure 6.1f).

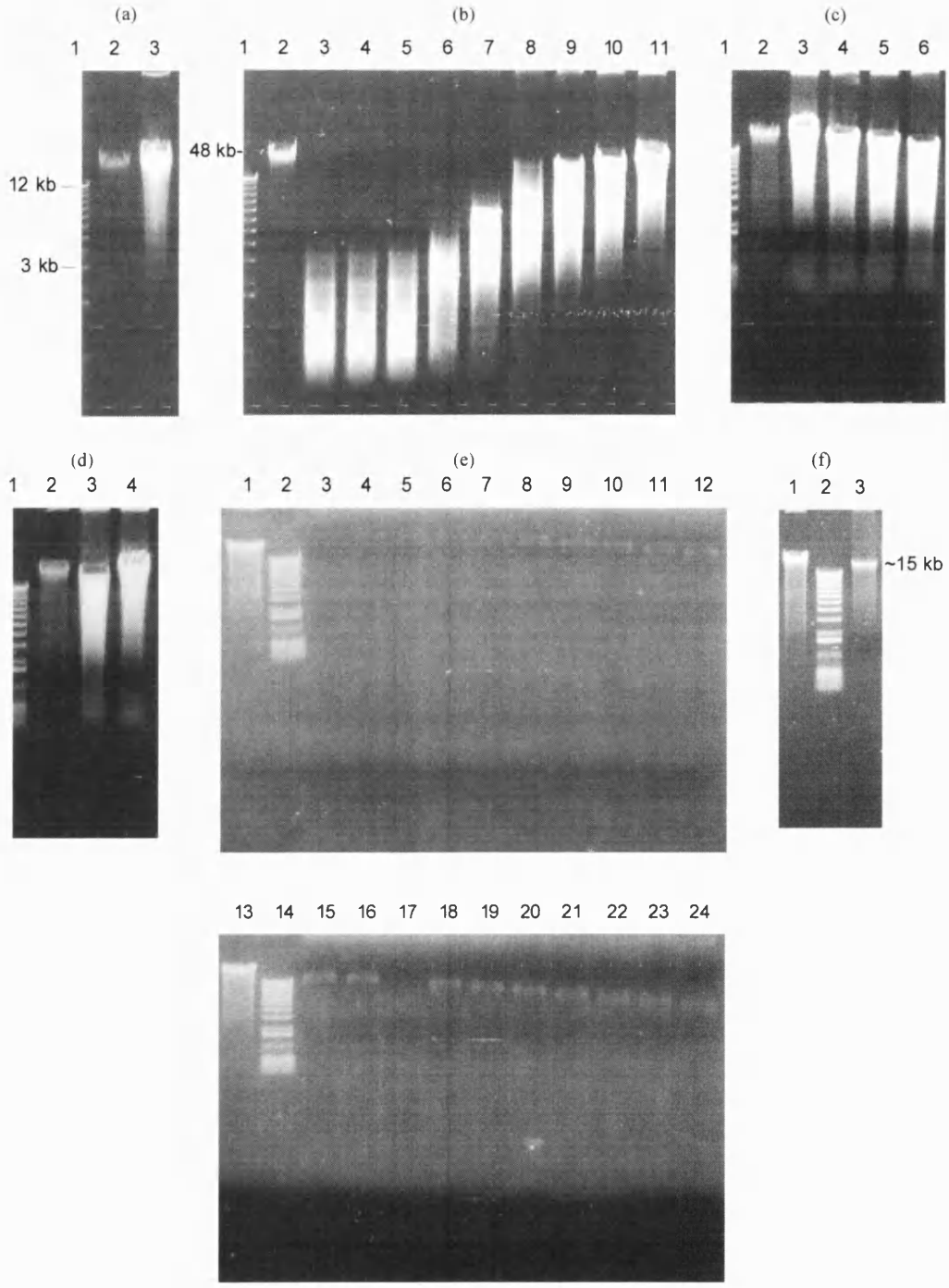


Figure 6.1 Preparation of *T. natronophilum* gDNA for the creation of a library in λ bacteriophage

(a) gDNA sample. Lane 1, 1 kb ladder, 2, λ DNA uncut, 3, *T. natronophilum* gDNA. (b) Pilot digestion with *Sau* 3AI. Lane 1, 1 kb ladder, 2, λ DNA uncut, 3 - 11, 10 μl of 20 μl reaction volume containing 18 μg of *T. natronophilum* gDNA and 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.040 and 0 U of *Sau* 3AI. (c) Scale-up digestion. Lane 1, 1 kb ladder, 2, λ DNA uncut, 3, *T. natronophilum* gDNA uncut, 4 - 6, 10 μl of 200 μl reaction volume containing 180 μg of *T. natronophilum* gDNA and 0.175, 0.35, 0.7 U of *Sau* 3AI. (d) Pool of *Sau* 3AI restricted fragments. Lane 1, 1 kb ladder, 2, λ DNA uncut, 3, 10 μl of pooled sample, 4, *T. natronophilum* gDNA uncut. (e) Analysis of size fractionation of *Sau* 3AI restricted gDNA fragments. Lanes 1 and 13, λ DNA uncut, 2 and 14, 1 kb ladder, 3 - 12 and 14 - 24, fractions 1 - 20. (f) Pooled fractions from size fractionation. Lane 1, λ DNA uncut, 2, 1 kb ladder, 3, 5 μl of pooled sample containing 15 kb *Sau* 3AI restricted gDNA fragments.

6.4.2 Design of oligonucleotide probes for pullulanase and α-amylase from *T. natronophilum*

Oligonucleotides used for probing the gDNA library were designed using the amino acid sequences previously determined. In order to reduce the possible number of non-specific hybridisations to the gDNA library the following measures were taken. Firstly, oligonucleotides were synthesised corresponding to those amino acids within the protein sequence that possess the least number of codons. Secondly, certain areas of degeneracy were resolved to some degree by the use of a codon usage table determined for *Thermotoga maritima*, using sequences available at that date (Figure 6.2 and Table 6.1).

6.4.3 Screening of the gDNA library using the oligonucleotide probes

Although screening of the *T. natronophilum* gDNA library did provide positive plaques in the primary screens, when the λ clones responsible were carried over to secondary screens they no longer hybridised with the probes, even though growth of the clones was apparent.

To increase the number of λ clones in the screening process a second library was prepared using the same 15 kb *Sau* 3AI cut gDNA fragment sample, but doubling the amount of each component in the ligation reaction. However, the same result was encountered upon screening the gDNA library.

Pullulanase

(a) Y I G D G A W E A V L E G D D E G X F Y R

(b) TyrIleGlyAspGlyAlaTrpGluAlaValLeuGluGlyAspAspGluGlyXXXPheTyrArg

(c) 2 3 4 2 4 4 1 2 4 4 6 2 4 2 2 2 4 X 2 2 6

(d) TAYATHGGNGAKGGNGCNTGGGARGCNGTNYTNGARGGNGAKGAKGARGGNXXXTTYTAYMGN

(e) TAYAT**A**GG**W**GA**T**GG**W**GCNTGG

α-Amylase

(a) I G L P S V M T E P W N P I G G S N W I F D M M L I R

(b) IleGlyLeuProSerValMetThrGluProTrpAsnProIleGlyGlySerAsnTrpIlePheAspMetMetLeuIleArg

(c) 3 4 6 4 6 4 1 4 2 4 1 2 4 3 4 4 6 2 1 3 2 2 1 1 6 3 6

(d) ATHGGNYTNCCNWSNGTNATGACNGARCCNTGGAAYCCNATHGGNGGNWSNAAYTGGATHTTYGAKATGATGYTHATHMGN

(e) AA**C**TGGAT**A**TTYGA**T**ATGATG

Figure 6.2 Oligonucleotide probe design from known protein sequence for the hydrolases from *T. natronophilum*

Known protein sequence expressed as single letter code (a) and three letter code (b). (c) Denotes the number of codons responsible for the relevant amino acid. (d) Shows the degenerate DNA sequence and (e) shows the sequence of the oligonucleotides. The degeneracies in bold in the oligonucleotides have been resolved using a codon usage table created using nucleotide sequences available for *T. maritima*.. Unknowns are denoted by X. The degenerate nucleotide IUB codes used are M (A/C), R (A/G), W (A/T), S (C/G), Y (C/T), K (G/T), H (A/C/G) and N (A/C/G/T).

Amino acid	Anti-codon	Frequency	Preferred anti-codon	Amino acid	Anti-codon	Frequency	Preferred anti-codon
Phe	TTT	0.44		Tyr	TAT	0.52	
	TTC	0.56	TTY		TAC	0.48	TAY
Leu	TTA	0.09		Stop	TAA	0.39	TRA
	TTG	0.20		Stop	TAG	0.19	TRA
	CTT	0.21		His	CAT	0.38	
	CTC	0.26			CAC	0.62	CAC
	CTA	0.04		Gln	CAA	0.35	
	CTG	0.19	YTB		CAG	0.65	CAG
Ile	ATT	0.24		Asn	AAT	0.34	
	ATC	0.25			AAC	0.66	AAC
	ATA	0.51	ATA	Lys	AAA	0.48	
Met	ATG	1.00	ATG		AAG	0.52	AAR
Val	GTT	0.26		Asp	GAT	0.67	
	GTC	0.18			GAC	0.33	GAT
	GTA	0.19		Glu	GAA	0.70	
	GTG	0.36	GTN		GAG	0.30	GAA
Ser	TCT	0.17		Cys	TGT	0.35	
	TCC	0.11			TGC	0.65	TGC
	TCA	0.20		Stop	TGA	0.42	TRA
	TCG	0.18	WSN	Trp	TGG	1.00	TGG
Pro	CCT	0.32		Arg	CGT	0.06	
	CCC	0.18			CGC	0.02	
	CCA	0.23			CGA	0.15	
	CCG	0.27	CCN		CGC	0.07	AGR
Thr	ACT	0.15		Ser	AGT	0.17	
	ACC	0.24			AGC	0.16	WSN
	ACA	0.27		Arg	AGA	0.46	
	ACG	0.34	ACN		AGG	0.25	AGR
Ala	GCT	0.25		Gly	GGT	0.28	
	GCC	0.21			GGC	0.09	
	GCA	0.28			GGA	0.44	
	GCG	0.26	GCN		GGG	0.19	GGW

Table 6.1 Anti-codon usage table for *T. maritima*

The results were calculated using the program CODONUSAGE in the GCG package (see section 5.1). The *T. maritima* sequences used were triose isomerase homologue (L27492), putative Rep protein gene (L19928), Hu DNA-binding protein gene (L23541), DNA repair protein (recA) gene (L23425) and rho gene (L27279). The degenerate nucleotide IUB codes used are R (A/G), W (A/T), S (C/G), Y (C/T), B (C/G/T) and N (A/C/G/T).

6.5 DISCUSSION

The λ libraries were of a sufficient quality for screening purposes. The degree of certainty that a library contains the gene of interest can be calculated using the following formula devised by Clark and Carbon [1976].

$$P = 1 - (1 - f)^N \qquad \text{Equation 6.1}$$

where,

P = probability that the library contains the target DNA

f = the fraction of total genome used as a source for each fragment

N = number of transformants

However in this case, f' rather than f would be more appropriate as the size of the target gene is taken into consideration.

$$f' = [1 - (x/L)](f) \qquad \text{Equation 6.2}$$

where,

x = length of target DNA

L = length of cloned DNA fragments

thus, the equation to be used is,

$$P = 1 - (1 - f')^N \qquad \text{Equation 6.3}$$

Using Equation 6.3 and assuming that both the α-amylase gene and pullulanase gene are the same size and that they consist of approximately 2.25 kb and that each transformant was unique, the probabilities for each library were calculated. The genome size of *T. natronophilum* was taken to be same size as the genome of *T. maritima* (1,100 kb). On average 2400 plaque forming units (pfu) were screened from the first library, and a much greater number of pfu were screened with the second library (approximately 8000). The probability of finding the target DNA within either of these libraries was calculated at 0.99999999999, a value of 9 recurring to 40 decimal places was calculated for the second library. Taking this into consideration it was apparent that a new approach was needed to probe the genes responsible for the α-amylase and pullulanase. The problem with the current probes would seem to be their small size combined with degeneracy, causing non-specific binding. Also, with the knowledge of the actual DNA sequence for the α-amylase protein fragment which was elucidated from PCR amplifications preceding this work (Chapter 7), it is apparent that there is an incorrect base in the α-amylase probe, which would undoubtedly have an effect on annealing to the correct sequence within the library during hybridisation (Figure 6.3).

6.6 CONCLUSIONS

The *T. natronophilum* gDNA library constructed in λ bacteriophage is adequate for screening for any genes that are encoded within the genome. However, the method of probing for the pullulanase and α-amylase genes needs to be redeveloped in order to minimise non-specific binding and the spurious results associated with it.

The next chapter deals with the steps taken to achieve a more specific probe and with subsequent screening attempts.

CHAPTER 7

Creation of a gene-specific probe using PCR

7.1 INTRODUCTION

Following unsuccessful attempts to locate and isolate the genes responsible for the pullulanase and α-amylase from *Thermopallium natronophilum*, it was apparent that a more specific probe was required for these purposes. Due to the lack of time available and the quality of protein sequence, it was decided to concentrate on one of the hydrolases, the α-amylase.

This chapter deals with the production of a more specific probe and subsequent screening attempts to isolate the gene responsible for the α-amylase.

7.2 MATERIALS

The random primed labelling kit, High prime®, was purchased from Boehringer Mannheim (Mannheim, Germany).

Other materials and their suppliers mentioned in this chapter are listed in chapter 2.

7.3 METHODS

7.3.1 Labelling of the probe using High prime®

High prime® is based on the "random primed" method developed by Feinberg and Vogelstein [1983, 1984], where any oligonucleotide can be labelled. A complementary DNA strand is synthesised by Klenow polymerase using the 3' termini of the random hexanucleotides in the reaction mixture as primers. Modified dCTP (*i.e.* labelled with ^{32}P) present in the reaction is incorporated into the newly synthesised complementary DNA strand. The labelling procedure was carried out as per manufacturer's instructions.

Other procedures mentioned in this chapter are outlined in chapter 2.

7.4 RESULTS

7.4.1 PCR amplification of 81 bp fragment

7.4.1.1 Design of the PCR primers

Oligonucleotide PCR primers were designed for the amplification of the nucleotide sequence responsible for the amino acid sequence of the α-amylase fragment, previously elucidated (Figure 7.1). It was not necessary to try to resolve any of the redundancies within the primers as a PCR product of known size was expected. The primers were designed in such a manner that they encoded those amino acids with single codons, at the 3' end, which crucially has to be true to the target sequence to be amplified. This also negates having to use more redundancies than necessary, thus reducing the number of non-viable primers in the amplification reaction.

7.4.1.2 Initial amplification reactions

Initial experiments using the above primers under normal PCR conditions, that is 2 mM $MgCl_2$, gave a broad range of PCR products, none of which were the correct size of approximately 81 bp. It was clear that the thermal cycling conditions for the amplification reaction were adequate from the number of products seen, but the components within the mixture needed to be optimised. The effect of $MgCl_2$ concentration on the amplification was studied as it is known to increase the melting temperature of primers, causing more rigid annealing to template DNA [McPherson, 1991]. As primer CRT04 has a low melting temperature of 34.4 °C compared with 53.2 °C for CRT03, it seemed likely that absence of the 81 bp PCR product is due this low melting temperature. Concentrations from 2 to 10 mM final concentration were studied (Figure 7.2).

An 81 bp product was produced at increasing amounts from 2 to 10 mM $MgCl_2$, but amounts were minute and the reaction components needed to be further optimised. Because of the low melting temperature, CRT04 was redesigned to incorporate a further 3 bases at the 3' end of the oligonucleotide. In order to reduce the number of non-viable primer variants within the reaction mixture, two primers were designed to eliminate the need for a redundancy within the primer (Figure 7.3). The redesigned primers both had melting temperatures above 40 °C, which was still not ideal, and therefore, amplification reactions were carried out in 10 mM $MgCl_2$.

Amplification reactions using CRT03 and either CRT07 or CRT08 gave contrasting results (Figure 7.4). CRT03 and CRT07 resulted in low amounts of a 81 bp PCR product compared with CRT03 and CRT08. This result would appear to resolve one of the degenerate bases

within the primer binding area, but to determine the whole sequence of the PCR product to confirm it correlates with the amino acid sequence, the PCR product needs to be prepared into a suitable form for subsequent sequencing.

7.4.2 Sub-cloning of the 81 bp PCR product and sequencing

Initial sequencing using the PCR product as template and the degenerate primers (CRT03 and CRT08) revealed that the PCR product contained the sequence of the gene fragment responsible for the α-amylase amino acid sequence (Figure 7.5). However, the sequences contained tentative bases and it was necessary to subclone the PCR product into a plasmid vector order to determine the precise sequence. The plasmid chosen was pGEMT (Figure 7.6) which allows direct ligation of the PCR product due to the independent addition of adenosine to the 3' termini by Taq polymerase, resulting in a one base overhang (sticky ends). The vector is supplied linearised with thymidine overhangs engineered onto the 3' termini.

4 out of 11 colonies screened from the transformation contained an insert of the correct size upon digestion with *Sph* I and *Nde* I (Figure 7.7). 2 of the 4 transformants were sequenced using universal sequencing primers (Figure 7.8 and 7.9).

α-Amylase

(a) I G L P S V M T E P W N P I G G S N W I F D M M L I R

(b) IleGlyLeuProSerValMetThrGluProTrpAsnProIleGlyGlySerAsnTrpIlePheAspMetMetLeuIleArg

(C) 5′**ATHGGNYTNCCNWSNGTNATG**3′-*CRT03*

5′ATHGGNYTNCCNWSNGTNATGACNGARCCNTGGAAYCCNATHGGNGGNWSNAAYTGGATHTTYGAKATGATGYTHATHMGN3′

3′TADCCNRANGGNSWNCANTACTGNCTYGGNACCTTRGGNTADCCNCCNSWNTTRACCTADAARCTMTACTACRADTADKCN5′

CRT04-3′**TACTACRADDATKCN**5′

Figure 7.1 Design of oligonucleotide PCR primers for α-amylase from *T. natronophilum*

Known protein sequence expressed as a single letter code (a) and three letter code (b). (c) Shows the derived nucleotide sequence for the protein sequence and the designed primers, shown in bold. The degenerate nucleotide IUB codes used are M (A/C), R (A/G), W (A/T), S (C/G), Y (C/T), K (G/T), H (A/C/T), D (A/G/T) and N (A/C/G/T).

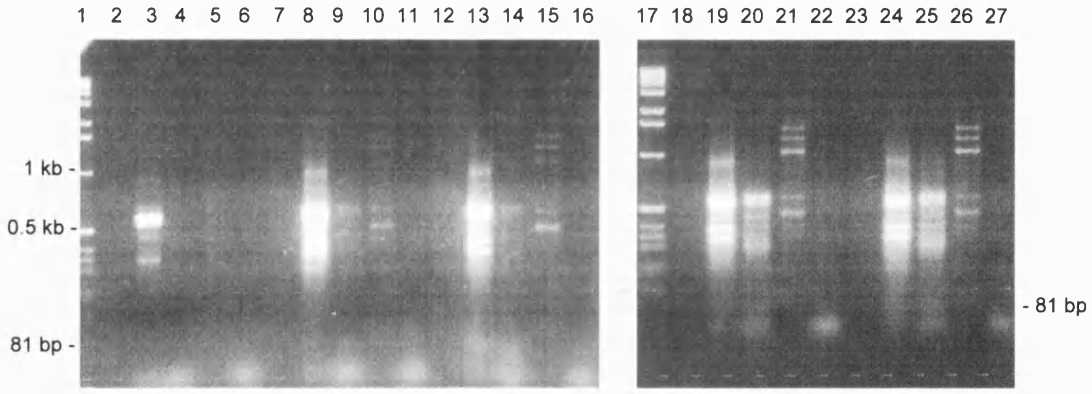


Figure 7.2 The effect of $MgCl_2$ concentration on the amplification of the α-amylase gene fragment
Lanes 1 and 17, 1 kb ladder, 2, 7, 12, 18 and 23, gDNA alone, 3, 8, 13, 19 and 24, CRT03 with gDNA, 4, 9, 14, 20 and 25, CRT03 and CRT04 with gDNA, 5, 10, 15, 21 and 26, CRT04 with gDNA, 6, 11, 16, 22 and 27, CRT03 and CRT04 alone. Lanes 2 - 6, 2 mM $MgCl_2$, Lanes 7 - 11, 4 mM $MgCl_2$, Lanes 12 - 16, 6 mM $MgCl_2$, Lanes 18 - 22, 8 mM $MgCl_2$ and Lanes 23 - 27, 10 mM $MgCl_2$. Samples were electrophoresced through a 2 % (w/v) agarose gel.

α-Amylase	(a)	D M M L I R
	(b)	AspMetMetLeuIleArg
	(c)	5′GAKATGATGYTHATHMGN3′
		3′CTMTACTACRADTADKCN5′
	(d)	*CRT04*- 3′TACTACRADDATKCN5′
	(e)	*CRT07*-3′CTATACTACRADDATKCN5′
		CRT08-3′CTGTACTACRADDATKCN5′

Figure 7.3 Redesign of PCR primer, CRT04
(a) Known protein sequence expressed as a single letter code and three letter code (b). (c) Shows the derived nucleotide sequence for the protein sequence. (d) Shows CRT04 primer and (e) shows the redesigned primers based on CRT04. The degenerate nucleotide IUB codes used are M (A/C), R (A/G), Y (C/T), K (G/T), H (A/C/T), D (A/G/T) and N (A/C/G/T).

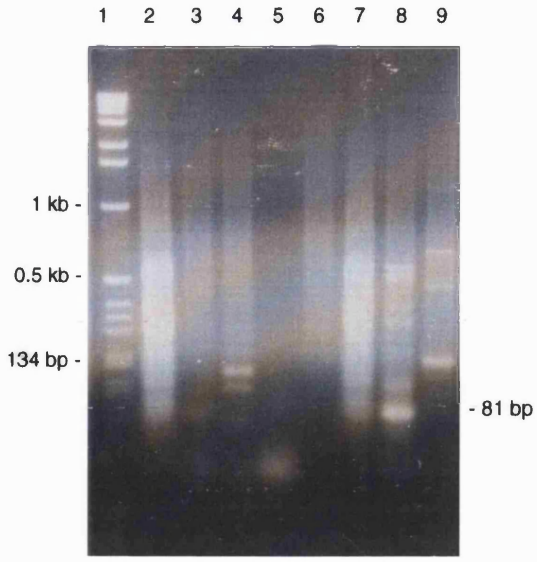


Figure 7.4 PCR amplification of α-amylase gene fragment

Lane 1, 1 kb ladder, 2 and 7, CRT03 with gDNA, 3, CRT03 and CRT07 with gDNA, 4, CRT07 with gDNA, 5, CRT03 and CRT07 and CRT08 alone, 6, gDNA alone, 8, CRT03 and CRT08 with gDNA, 9, CRT08 with gDNA. Samples were analysed on a 2 % (w/v) agarose gel.

(a) 5'CNTGGACCGCgGGggTcaAACTGGATATTTGACaTGATGCTCATAAGaA3'
XXXAspArgGlyGlySerAsnTrpIlePheAspMetMetLeuIleArg
X D R G G S N W I F D M M L I R

(b) 5'CTTTNAGAACACGAtgGGGTTCcAAGGTtCTGTCATAACgCAAGGAAAACCAATA3'
LysXXXPheValIleProAsnTrpProGluThrMetValCysProPheGlyIle
K X F V I P N W P E T M V C P F G I

(c) I G F P C V M T E P W N P I G G S N W I F D M M L I R

(d) I G L P S V M T E P W N P I G G S N W I F D M M L I R

Figure 7.5 Direct sequencing of 81 bp PCR product

(a) Shows the nucleotide sequence of the sense strand determined using CRT03 primer and the derived amino acid sequence. (b) Shows the nucleotide sequence of the anti-sense determined using CRT08 primer and the derived amino acid sequence. (c) Shows the overall amino acid sequence derived from the nucleotide sequences, those highlighted in red are from (a) and those highlighted in yellow are from (b). (d) Shows the amino acid sequence determined by protein sequencing. Residues in lower case represent tentative assignments.

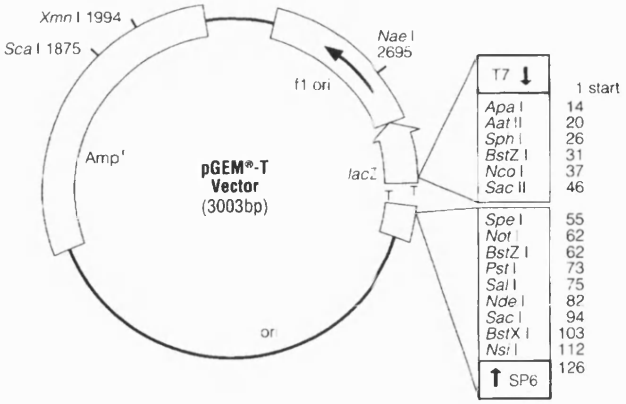


Figure 7.6 pGEMT Vector
(Reproduced from Promega catalogue, Madison WI, USA)

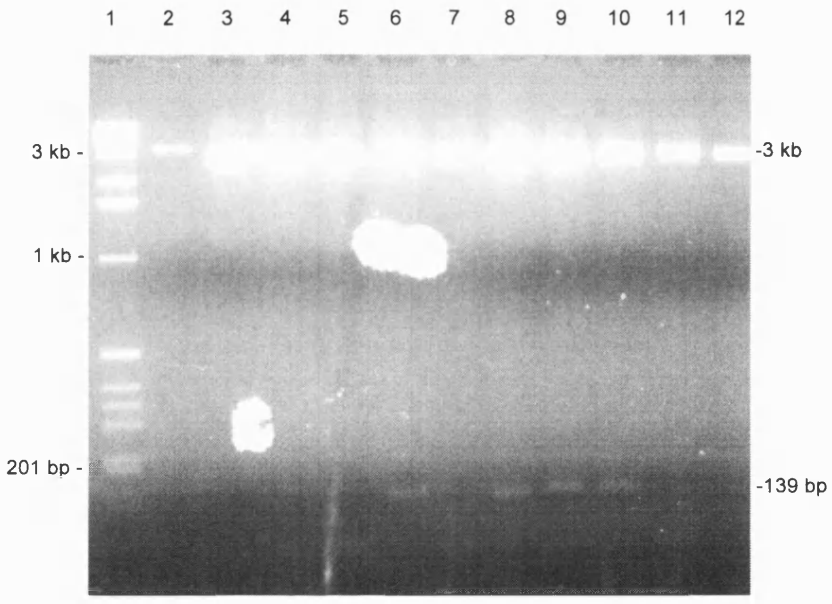


Figure 7.7 Restriction digest of transformants containing pGEMT
Lane 1, 1 kb ladder, Lanes 2 - 12, transformants 1 - 11. Digestions were carried out with *Sph* I and *Nde* and samples were analysed on a 2 % (w/v) agarose gel by electrophoresis.

SEQUENCING-5'CAGGAAACAGCTATGAC3'
REVERSE- 5'GTAAAACGACGGCCAGT3'

Figure 7.8 Composition of universa primers for pGEMT

Transformant 1

(a) 5′ATTGGGTTTCCGTGCGTCATGACAGAACCCTGGAACCCAATCGGTGGTTCTAACTGGATATTTGACATGATGCTAATCAGC3′

3′TAACCCAAAGGCACGCAGTACTGTCTTGGGACCTTGGGTTAGCCACCAAGATTGACCTATAAACTGTACTACGATTAGTCG5′

(b) IleGlyPheProCysValMetThrGluProTrpArgProIleGlyGlySerAsnTrpIlePheAspMetMetLeuIleSer

(c) I G **F** P **C** V M T E P W N P I G G S N W I F D M M L I **S**

Transformant 2

a *e*

b c *d* *f* *g* *hi*

(a) 5′ATTGGTTTTCCTTCTGTTATGACAGAACCCTGGAACCCAATCGGTGGTTCTAACTGGATATTTGACATGATGCTAATAAGA3′

3′TAACCAAAAGGAAGACAATACTGTCTTGGGACCTTGGGTTAGCCACCAAGATTGACCTATAAACTGTACTACGATTATTCT5′

(b) IleGlyPheProSerValMetThrGluProTrpArgProIleGlyGlySerAsnTrpIlePheAspMetMetLeuIleArg

(c) I G **F** P S V M T E P W N P I G G S N W I F D M M L I R

Expected

I G L P S V M T E P W N P I G G S N W I F D M M L I R

Figure 7.9 Sequence results from transformants

(a) Shows the determined nucleotide sequence. (b) and (c) Show the amino acids encoded by the nucleotide sequence in three letter and single letter codes, respectively. Discrepancies between derived amino acid sequence from the nucleotide sequence and those determined by protein sequencing (expected) are in bold. Underlined nucleotides represent those nucleotides found within the degenerate primer binding regions. Restriction sites are indicated by an italicised letter, *a*, *Bsa* JI, *b*, *Eco* RII, *c*, *Scr* FI, *d*, *Nla* IV, *e*, *Bsl* I, *f*, *Pfl* MI, *g*, *Drd* II, *h*, *Bsr* I and ,*i*, *Sfa* NI.

7.4.3 Re-screening of the gDNA library

Following the successful amplification and cloning of the nucleic acid responsible for the α-amylase fragment, a strategy was devised to locate a clone within the λ library which contains the α-amylase gene.

Random primed labelling was used to produce a probe from the 81 bp PCR product using the labelling kit, High prime® and [αP^{32}]dCTP.

Unlike screening using degenerate oligonucleotide probes, there were far more signals observed in the screens. Although it was obvious that the low stringency conditions (50 °C washes) were giving rise to numerous non-specific interactions between the λ clones and the random-primed probe, there were signals of much greater intensity which appeared promising (Figure 7.10).

When the membranes from the primary screen were re-washed at a higher temperature (55 °C) and re-exposed to x-ray film for a similar length of time the number of signals was reduced and a small number remained intense (Figure 7.11).

12 Plaque "plugs" were taken from the plates and carried forward to a secondary screen. Again the membranes from this secondary were probed using the same random-primed probe. The screen produced a range of results varying from weak or few signals to many and very intense signals (Figure 7.12). 1 Plaque "plug" was taken from 4 of the 12 plates screened. The plaques chosen were independent of any other signals or plaques to minimise the risk of contamination.

The recombinant λ DNA from the four plaques was extracted using λ MAXI DNA preparation kit. Recovery of the DNA was adjudged to be adequate when small samples were analysed by agarose gel electrophoresis (Figure 7.13).

Direct sequencing of the clones was attempted using one of the degenerate PCR primers (CRT08). Unfortunately, no sequence was returned, in an attempt to facilitate the sequencing reaction in the initial stages, the λ clone DNA was denatured by heating to 95 ° C for 10 min and snap-cooled on ice prior to the addition of CRT08 and the sequence reaction components. This attempt was also forlorn.

Non-degenerate primers were designed using the previously elucidated PCR sequence (Figure 7.14). Sequencing was again attempted on all 4 clones using primer CRT09 and CRT10 for clone 1. Again all of the sequencing reactions failed to produce any sequence, although, when universal primers were used sequence was obtainable.

In order to assess whether the 4 clones were individual clones or not, various restriction digests were undertaken. Upon digestion with the restriction endonucleases each clone displayed a similar restriction pattern, indicating that each clone contained the same DNA insert.

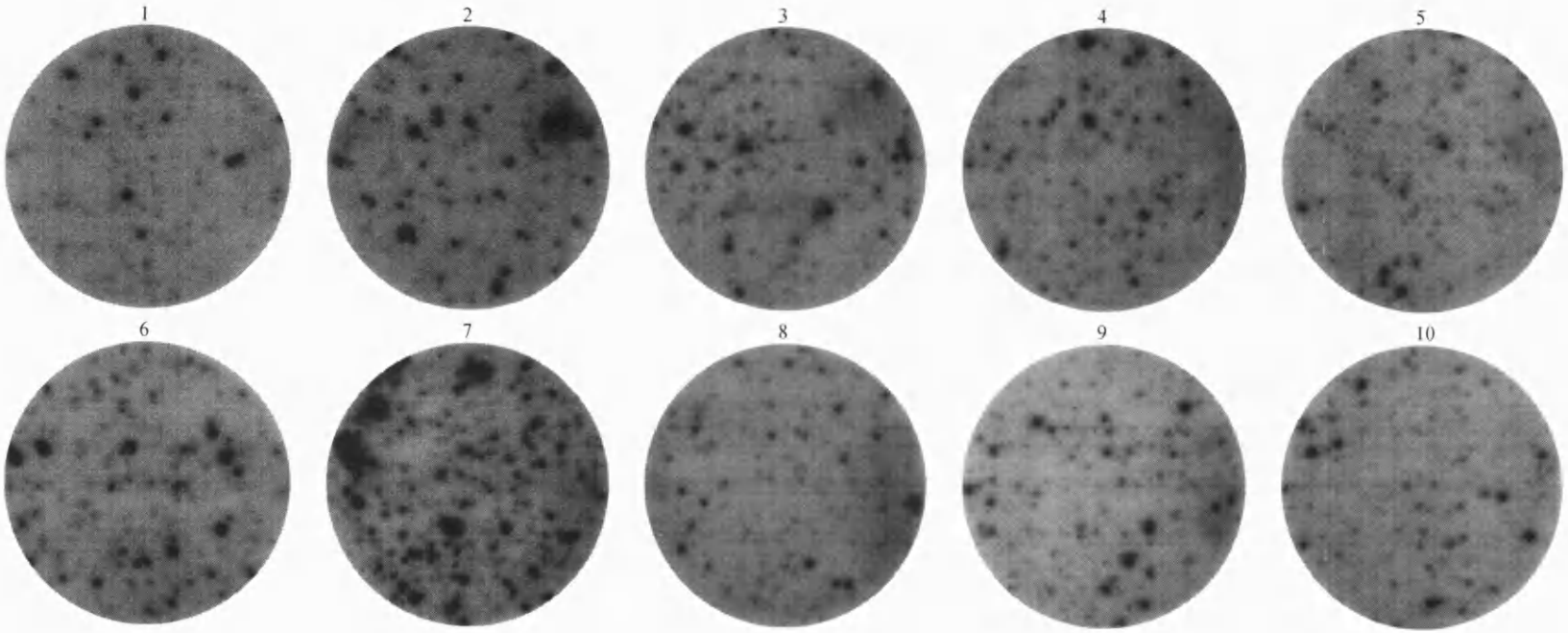


Figure 7.10 X-ray film exposed to primary screen performed under low stringency

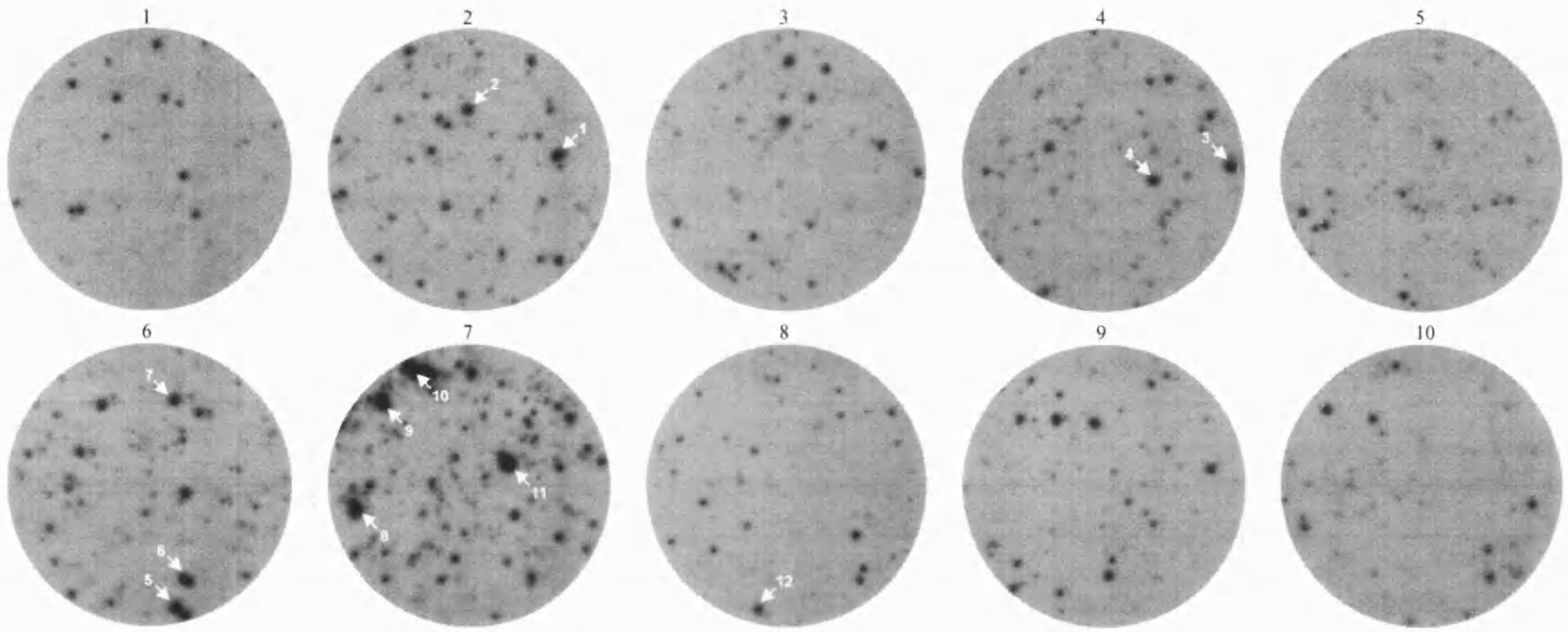


Figure 7.11 X-ray film exposed to primary screen performed under higher stringency

Plaques taken for the second screen are indicated with a numbered arrow, corresponding to the plate number in the second screen.

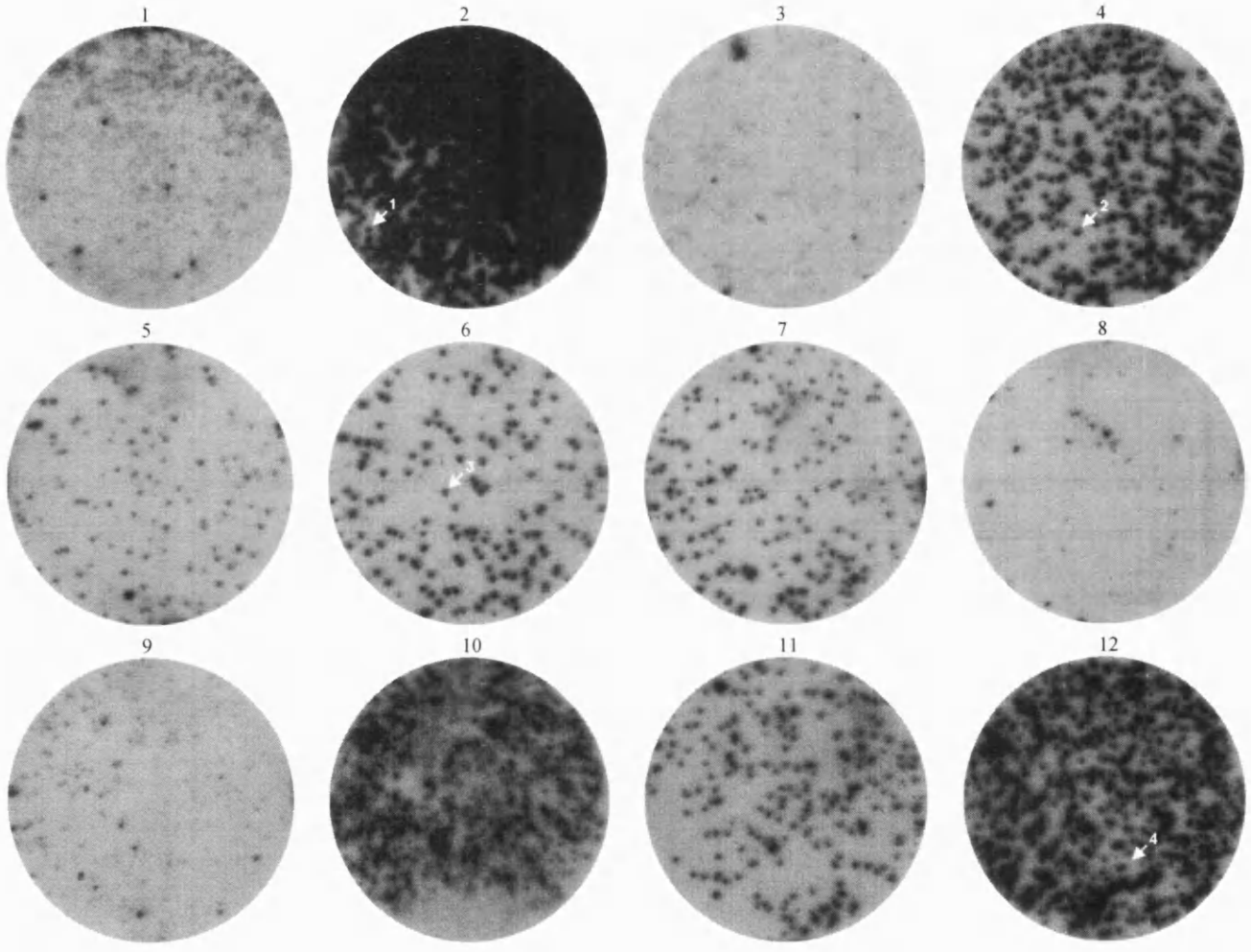


Figure 7.12 X-ray film exposed to secondary screen performed under high stringency
Plaques taken for subsequent analysis are indicated with a numbered arrow, corresponding to the clone number in subsequent procedures.

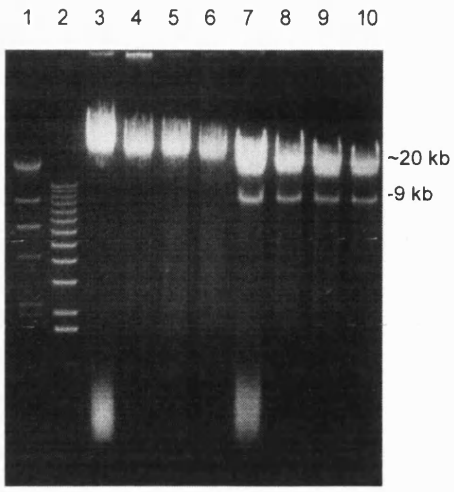


Figure 7.13 Preparation of λclone DNA

Lane 1, λ *Hind* III, 2, 1 kb ladder, 3 - 6, λ clones 1 - 4, 7 - 10, λ clones digested with *Sal* I. Samples were analysed on a 0.5 % (w/v) agarose gel.

(a)

CRT09-5′ATGACAGAACCCTGGAAC3′

CRT10-5′CATCATGTCAAATATCCA3′

(b)

EMBL3L (LEFT ARM)-5′GTGATGCCATGGTGTCCGACT3′

EMBL3R (RIGHT ARM)-5′CTCTCCAGAGGTTCATTACTG3′

Figure 7.14 Design of sequencing primers

7.4.4 Southern blot analysis of gDNA and λ clone

Difficulty in direct sequencing of inserts cloned into λ bacteriophage vectors has been encountered before within the research group, so the λ clone was persevered with.

Firstly, a Southern blot was performed on gDNA to assess the specificity of the probing procedure. Preparations of gDNA were individually digested with *Eco* RI, *Hind* III, *Nde* I, *Not* I, *Pst* I, *Sac* I and *Xba* I, and samples were analysed on a 0.7 % (w/v) agarose gel by electrophoresis (Figure 7.15a). The DNA fragments were transferred from the gel to a nylon membrane using Southern blotting. The membrane was probed with the random primed PCR product at 45 °C overnight and the membrane was washed the following day at 55 °C.

λ clone 1 was digested with *Eco* RI, *Spe* I and *Xho* I, both individually and together in double digests. Samples of each restriction digestion were analysed on a 0.7 % (w/v) agarose gel by electrophoresis (Figure 7.16a). The gel was treated in the same way as outlined for the gDNA Southern blot analysis.

The gDNA Southern blot yielded a number of signals (Figure 7.15b). Bands corresponding to gDNA and the PCR product gave intense signals. Much weaker signals were given by the bands from the digests compared to gDNA and PCR product signals (Figure 7.15c), this is due to the cumulative effect of non-specific and specific signals in the case of gDNA and the lower concentration of specific sites in the case of the PCR product.

The λ clone Southern blot gave rise to singular signals for each restriction digest. More promisingly, one of these signals matched-up with one of those seen in the gDNA Southern blot. This suggests that the signal seen with the λ clone blot is due to the insert rather than the λ vector itself (Figure 7.16), as the λ *Hind* III marker did not show any positive hybridisation with the probe.

The predicted size of the α-amylase gene is 2 - 2.5 kb and the maximum acceptable size insert for pUC18 is in the region of 6 kb. Taking these values into consideration, the *Eco* RI cut 4.5 kb fragment seemed an ideal candidate for sub-cloning.

In order to sequence the 4.5 kb nucleotide fragment, it was ligated into dephosphorylated *Eco* RI cut pUC18. The resulting construct was used to transform competent JM109 cells.

14 out of the 21 transformants were chosen to be cultured overnight and the plasmid DNA was extracted by the miniprep procedure. Upon digestion 11 of the 14 plasmids that grew appeared to carry a 4.5 kb insert (Figure 7.17).

Upon digestion with *Pfl* MI and *Eco* RI the plasmid yielded 3 fragments, one relating to the pUC18 vector (2.6 kb) and 2 from the insert (1.6 kb and. 2.9 kb). *Pfl* MI was used as a *Pfl* MI restriction site has been identified within the nucleotide sequence responsible for the α-amylase amino acid fragment. Because *Pfl* MI does not cut the vector DNA and the insert comes form a complete digestion with *Eco* RI, it appears that there is a single *Pfl* MI within the insert DNA, occurring 1.6 kb in from one end. A *Pfl* MI restriction site is also present in the nucleotide sequence responsible for the α-amylase fragment. Although this seemed promising, upon sequencing with primers designed using the PCR sequence, no sequence was returned. Again, sequence was obtainable when using universal primers. The primers designed from the PCR sequence were tested on the pGEMT clone carrying the 81 bp PCR product (pGEMT81) and sequence was obtainable.

Although sequencing failed using primers designed from the 81 bp PCR product sequence, a combination of universal primers and primers designed from the previous sequencing were used to sequence through the 1.6 kb *Pfl* MI restriction site. Unfortunately the sequence flanking the *Pfl* MI site did not correspond to the PCR product sequence.

On closer inspection of the restriction digest of λ clone 1 with *Eco* RI, there appeared to be two bands instead of a single band migrating at around 4.5 kb. Therefore, more λ clone 1 DNA was digested with *Eco* RI and the bands were excised and purified using the Geneclean® kit and ligated into pUC18, that had been prepared by restriction with *Eco* RI and dephosphorylated prior to purification by Geneclean®. JM109 cells were successfully transformed with the resulting ligation mixture. However, the transformants failed to produce any sequence when primers specific to the PCR sequence were used.

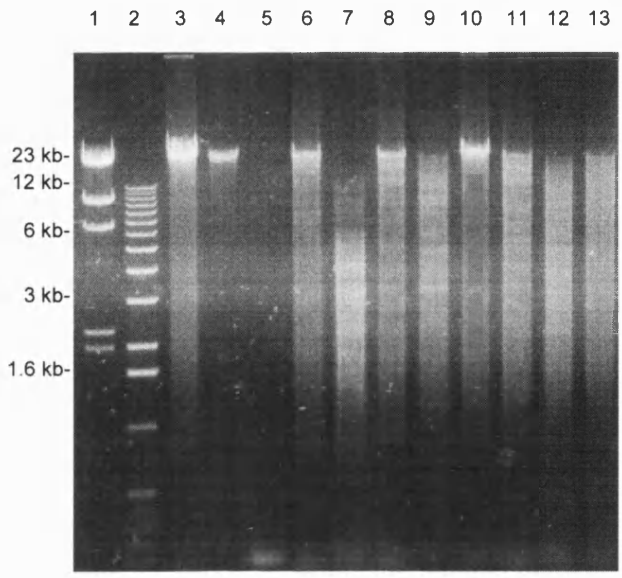
(c)

Lane	Description	Fragment size (kb)
3	gDNA	**> 12.0**
4	λ Clone 1	> 12.0*
5	81 bp PCR product	**81 (bp)**
6	gDNA - *Bam* HI	**> 12.0**, > 12.0, 7.7
7	gDNA - *Eco* RI	8.5, **5.2**, **4.5**, 4.0, 2.4, **1.3**
8	gDNA - *Hind* III	> 12.0 (x2), 9.4
9	gDNA - *Nde* I	> 12.0, **4.0**, **2.1**
10	gDNA - *Not* I	**> 12.0**
11	gDNA - *Pst* I	7.7, **1.5**, **1.2**
12	gDNA - *Sac* I	11.2, 4.6
13	gDNA - *Xba* I	N/A

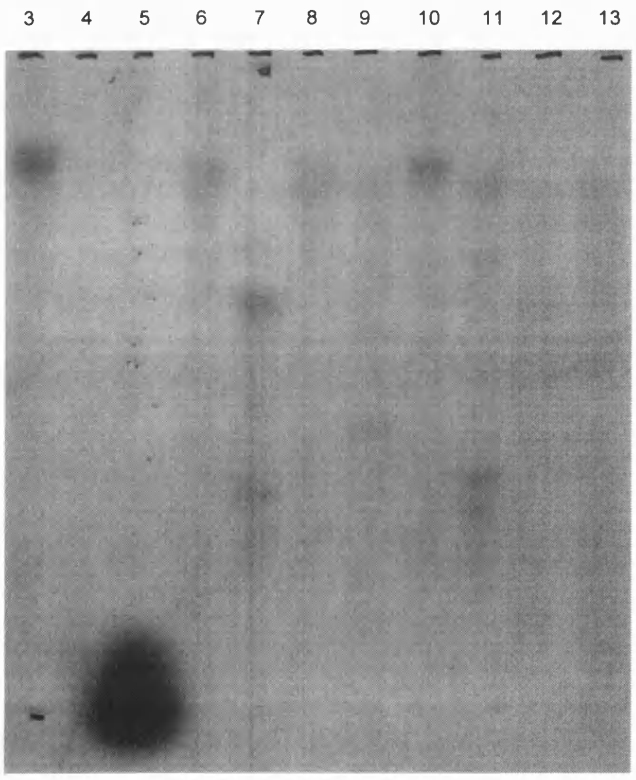
Figure 7.15 Southern blot analysis of gDNA digests

(a) Agarose gel showing the restriction digests. Lane 1, λ *Hind* III, 2, 1 kb ladder, 3, gDNA, 4, λ clone 1, 5 -13, gDNA digested with, *Bam* HI, *Eco* RI, *Hind* III, *Nde* I, *Not* I, *Pst* I, *Sac* I and *Xba* I. (b) Exposed x-ray film. Lanes as for (a). (c) Size of DNA fragments responsible for signals seen on the x-ray film. * Very feint band.

(a)


1 2 3 4 5 6 7 8 9 10 11 12 13
23 kb-
12 kb-
6 kb-
3 kb-
1.6 kb-

(b)


3 4 5 6 7 8 9 10 11 12 13

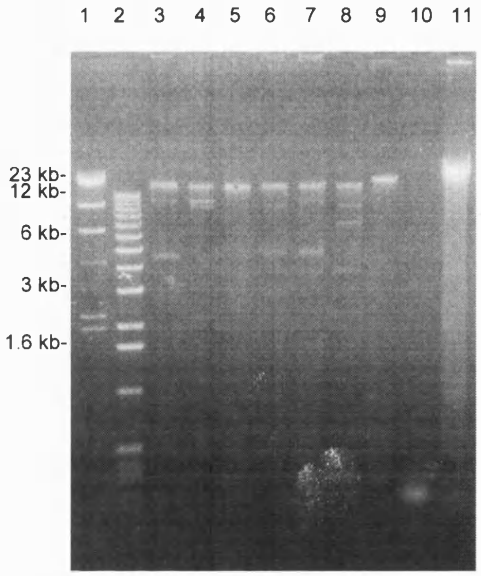
(c)

Lane	Description	Fragment size (kb)
3	λ Clone 1 - *Eco* RI	**4.5**
4	λ Clone 1 - *Spe* I	**10.5**
5	λ Clone 1 - *Xho* I	**> 12.0**
6	λ Clone 1 - *Eco* RI/*Spe* I	**4.5**
7	λ Clone 1 - *Eco* RI/*Xho* I	**4.5**
8	λ Clone 1 - *Spe* I/*Xho* I	**9.0**
9	λ Clone 1	> 12.0
10	81 bp PCR product	**81 (bp)**
11	gDNA	**> 12.0**

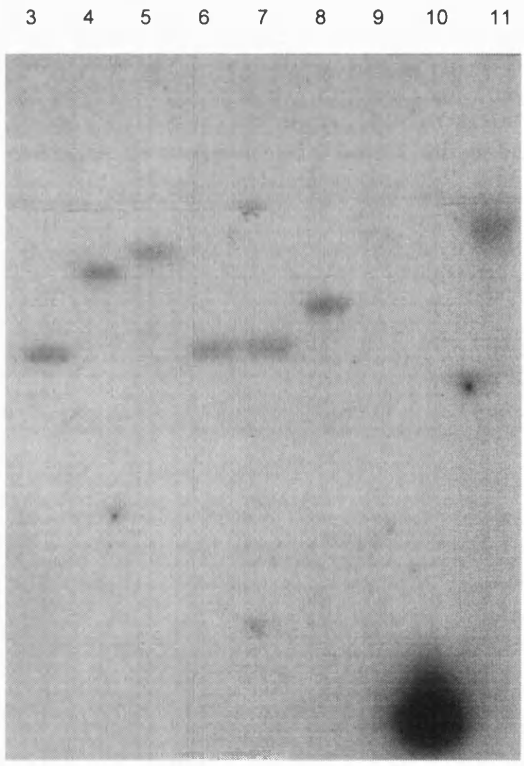
Figure 7.16 Southern blot analysis of clone 1 digests

(a) Agarose gel showing the restriction digests. Lane 1, λ *Hind* III, 2, 1 kb ladder, 3 - 8, λ clone 1 digested with, *Eco* RI, *Spe* I, *Xho* I, *Eco* RI/*Spe* I, *Eco* RI/*Xho* I, *Spe* I/*Xho* I, 9, λ clone 1, 10, 81 bp PCR product and 11, gDNA. (b) Exposed x-ray film. Lanes as for (a). (c) Size of DNA fragments responsible for signals seen on the x-ray film.

(a)



(b)



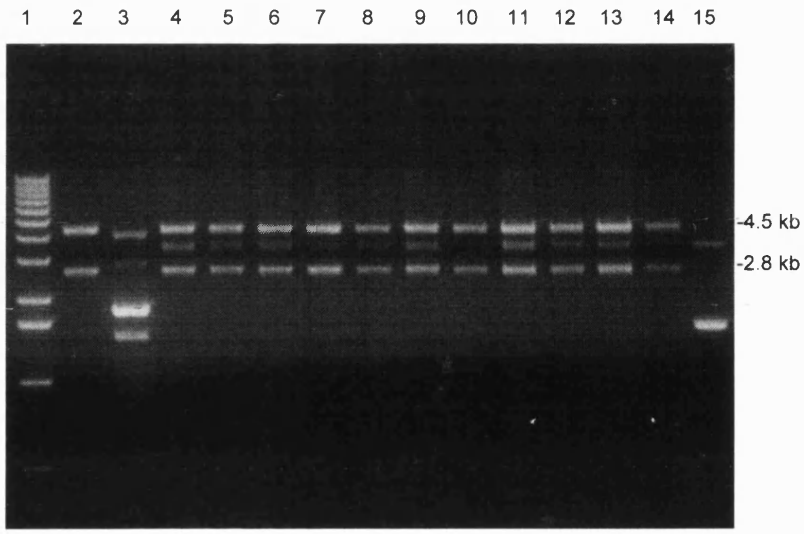


Figure 7.17 Restriction digestion of pUC18 transformants with *Eco* RI
Lane 1, 1 kb ladder, 2 - 15, *Eco* RI digested plasmid DNA extracted from transformant 1 -14.

7.5 DISCUSSION

Due to the lack of time available and the quality of the protein sequences, it was decided to overlook the pullulanase and concentrate efforts on cloning the α-amylase from *T. natronophilum*.

Numerous attempts were made to locate the λ clone containing the α-amylase gene using a partially degenerate oligonucleotide primer, which was 3' end labelled with [γP^{32}] dATP. This failed to give any reasonable or sustainable signals when carried through the screening procedure.

PCR was used to amplify a gene specific product which was used to produce a probe using a kit from which much success had been gained within the group. Upon screening the λ library the probe produced a large number of signals; in fact most plaques gave a signal albeit of differing intensity. It was, of course, unlikely that each of these plaques contain the λ clone encoding the α-amylase gene. It was clear that the low wash temperature of 50 °C was not eliminating non-specific interactions between the probe and the gDNA library. Washing at the higher temperature of 55 °C resulted in either the complete elimination of signals or a reduction of intensity in others. Even with the higher washing temperatures a high number of signals were still apparent. However, it was clear that of the remaining signals some remained intense when compared with the others. Even when these intense signals were carried over to the secondary screen the signs were promising. However, subsequent sequencing attempts failed. Problems had been encountered with the sequencing from λ libraries in the past so the λ clone was persevered with. After subsequent sub-cloning and sequencing it was clear that the λ clone did not contain the target α-amylase gene.

This once again outlined a problem with the labelling procedure. The same labelling kit, High prime®, had been used by several members of the research group for oligonucleotides as small as 114 bp with success. Although the product instructions claimed that a 200 bp fragment is as efficiently labelled as a 15 kb fragment of DNA, it failed to specify any limitations for the use of the kit and though the, kit worked when using a 114 bp fragment it was clearly not working for the 81 bp PCR product. The problem lies within the principle of how the kit works. The kit works on the principle of random priming, where degenerate hexanucleotides anneal to the denatured DNA probe and are extended by Klenow polymerase, with labelling achieved by the incorporation of [αP^{32}] dCTP. The problem encountered in the current work is almost certainly due to many of the hexanucleotides annealing to the same strand of DNA, thus producing many small products rather than the full 81 bp nucleotide probe, resulting in less specific probes.

7.6 CONCLUSIONS

Although the probe produced more signals than the 3' end labelled oligomeric probe, the probe possessed a high degree of non-specificity. The non-specificity of the probe has so far produced many signals during screens and do little to aid locating the α-amylase gene.

The next chapter deals with the production of a more specific probe for the α-amylase gene fragment and subsequent screening and blotting analysis.

CHAPTER 8

Optimisation of the probe labelling procedure

8.1 INTRODUCTION

So far, attempts to locate the α-amylase gene have failed. This seems to be due to the low specificity of the probe used to hybridise to the gDNA library. Two labelling procedures have been investigated, the procedure, which uses the High prime® kit, being more successful than the 3' end labelling method. This chapter addresses the specificity problems by developing a labelling procedure where the whole nucleotide sequence of the PCR product is labelled using a PCR based approach.

8.2 MATERIALS

All materials and their suppliers mentioned in this chapter are listed in chapter 2.

8.3 METHODS

8.3.1 Production of α-amylase probe

PCR amplification of the 81 bp gene fragment from the pGEMT clone containing the 81 bp PCR product was carried out according to the procedure outlined in section 2.2.7. The PCR products were analysed on a 2 % (w/v) agarose gel and the band corresponding to the 81 bp PCR product was excised and then extracted from the agarose gel slice using the MERmaid® kit, as per manufacturer's instructions. 50 ng of the 81 bp PCR product was then used as template for a second PCR reaction. The second PCR reaction was identical to the first with the exception of dCTP being replaced with 3000 μCi of [αP^{32}]dCTP. The reaction products were separated from the un-incorporated radioactive nucleotides by centrifugation through gel filtration media (see section 2.2.18). Typically, 5 μl of the resulting mixture was found to be sufficient for each plate being screened.

8.3.2 Stripping of hybridised membranes

The membranes were stripped by washing in 0.4 M NaOH for 30 min at 45 °C, followed by a further wash for 15 min at 45 °C in a solution of 0.1X SSC, 0.1 % (w/v) SDS and 0.2 M Tris-HCl, pH 7.5. The membranes were then ready to be hybridised with a new probe.

Other procedures mentioned in this chapter are outlined in chapter 2.

8.4 RESULTS

8.4.1 Production of a radiolabelled probe using PCR

Using the pGEMT81 clone as template, a PCR reaction was carried out to incorporate [αP^{32}] dCTP into the 81 bp PCR product, again using CRT03 and CRT08 as primers. As the procedure involves radioactive material it was not possible to analyse the products from this PCR reaction on an agarose gel by electrophoresis. PCR reactions using the pGEMT81 clone and primers CRT03 and CRT08 had previously been carried out and the sole product appeared to be an 81 bp fragment (Figure 8.1).

8.4.2 Re-screening of the gDNA library

10 plates were prepared for screening by the same procedure mentioned earlier in this work. To demonstrate the improvement in specificity of the probe, the subsequent blots made from the plates were hybridised with the probe produced by the PRIME® kit. The membranes were hybridised at 45 °C with the probe and following a 16 h incubation, the membranes were washed at 65 °C. It is clear from the resulting autoradiograph that the probe is not as specific as one would like it to be (Figure 8.2). Not only is the probe not wholly specific, there also appears to be signals which do not correspond to plaques when referred back to the original plate. This is more than likely to be due to the presence of un-incorporated radiolabelled nucleotide. The nucleotides were not removed as it was deemed "unnecessary" by the manufacturer's of the High prime® kit. However, the opposite appears to be the case in this instance.

The membranes were stripped and re-probed using the probe labelled by the PCR method, hybridisation and washing being carried out under the same conditions as for the previous screen. In contrast to the previous screen, there is a vast reduction in the number of signals. Additionally, the signals corresponded to plaques observed on the originating top agar plates (Figure 8.3)

When the 4 plaques identified in Figure 8.3 were used to infect *E.coli* for a secondary screen using the same probe, the results were promising (Figure 8.4). 4 clones from this second screen were then prepared for maxiprep DNA extraction using the Qiagen Lambda MAXI kit. However, even after several attempts it was not possible to prepare DNA from clone 2 as no growth occurred and no DNA was observed after the purification procedure.

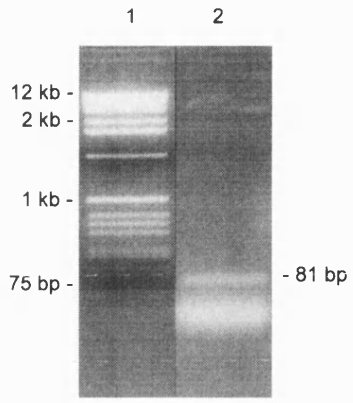


Figure 8.1 Analysis of PCR products using primers CRT03 and CRT08 and pGEMT81 clone as template Lane1, 1 kb ladder & 2, PCR products using CRT03 & CRT08 with pGEMT81 clone as template.

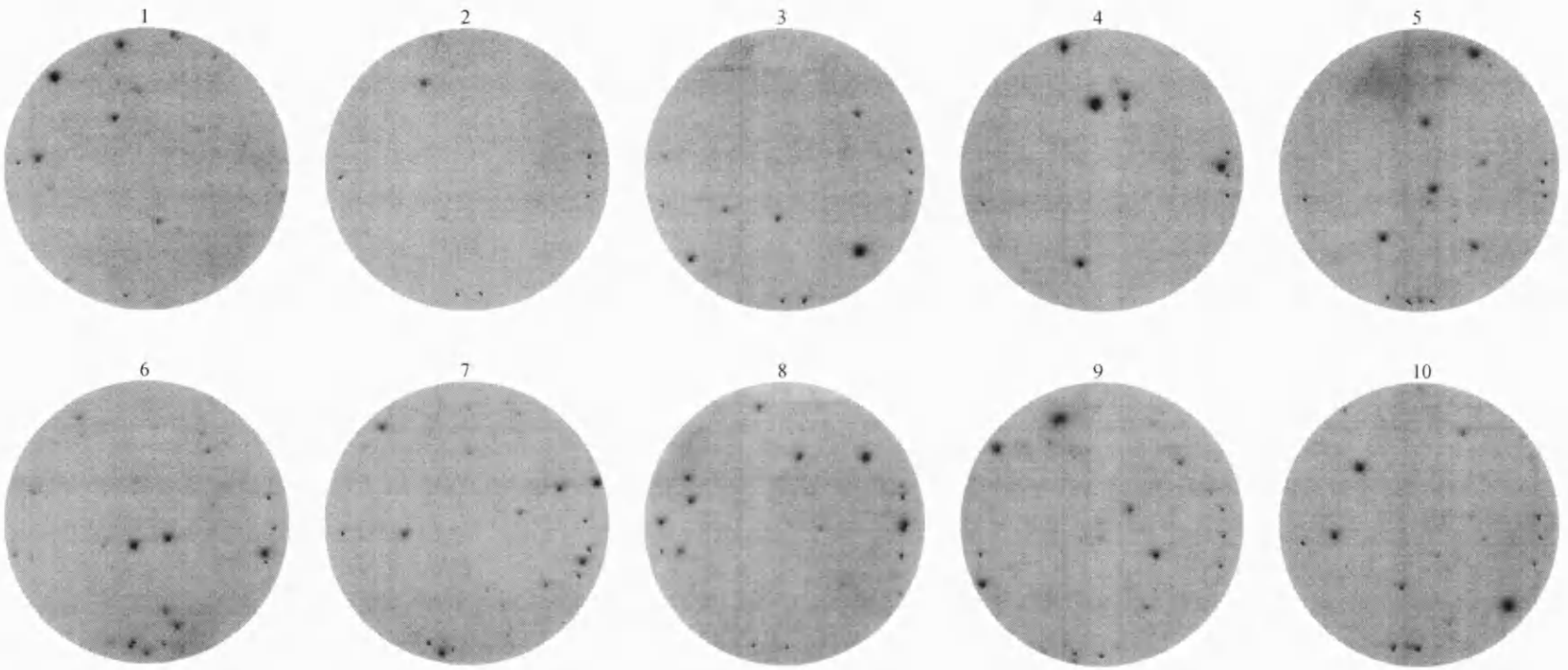


Figure 8.2 X-ray film from primary screen using a probe produced using the HIGH PRIME® kit
The sharply defined dots represent pen marks, used to align autoradiographs with the originating top-agar plates.

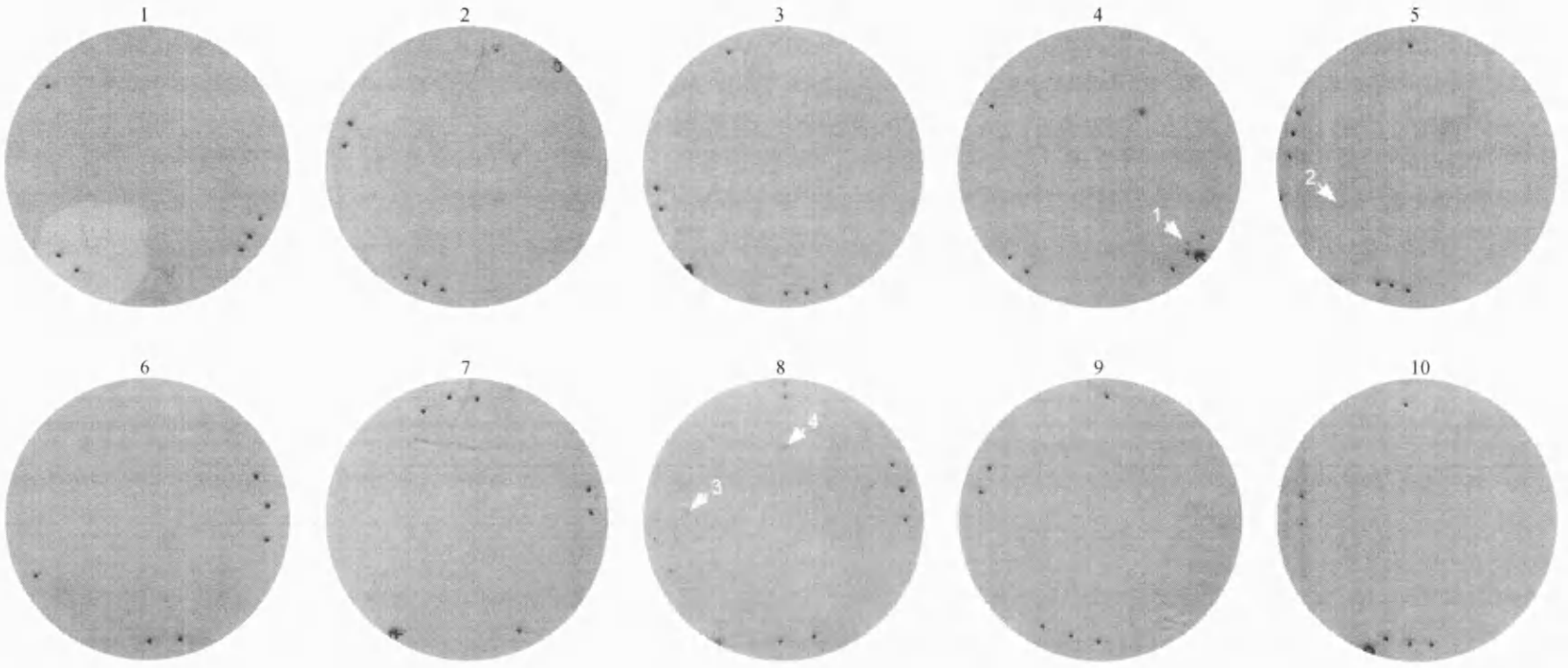


Figure 8.3 X-ray film from primary screen probed using the PCR based labelling procedure

Plaques taken for the second screen are indicated with a numbered arrow, corresponding to the plate number in the second screen. The sharply defined dots represent pen marks, used to align autoradiographs with the originating top-agar plates.

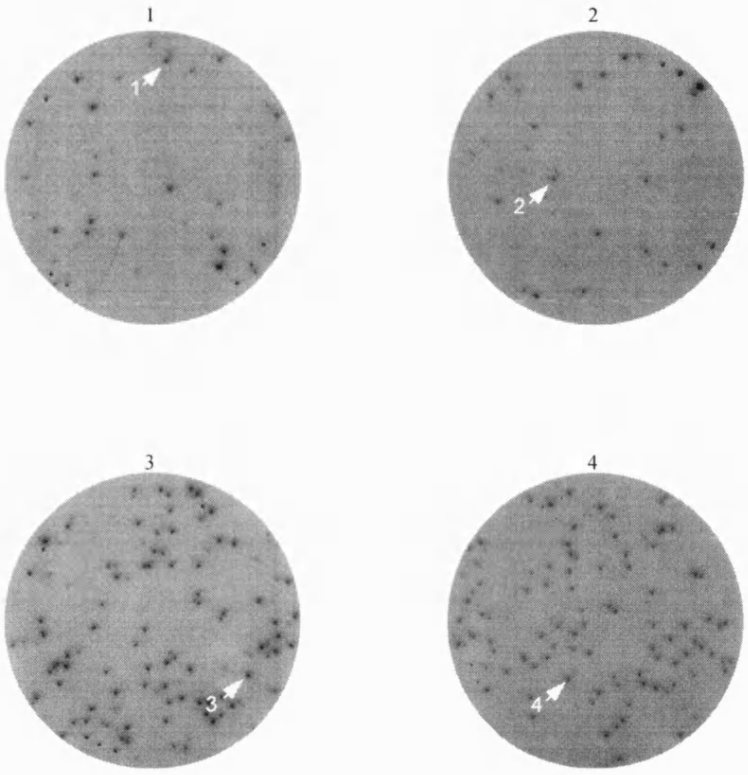


Figure 8.4 X-ray film from secondary screen probed using the PCR based labelling procedure
Plaques taken for further analysis are indicated with a numbered arrow, corresponding to the clone number in subsequent analysis.

The 3 clones that did provide DNA were judged to be different by subsequent restriction analysis. However, similar to previous attempts, no sequence was obtainable using the PCR based primers. A Southern blot was performed on a single digest of each of the 3 clones. This analysis resulted in 3 different sized *Pst* I cut fragments for the 3 clones. Curiously, the signals appeared to be weak compared to the previous screening attempts. Additionally, the DNA size markers appeared to produce signals (Figure 8.5).

When the procedure was repeated using a gDNA Southern blot and the 81 bp PCR product as template in the "labelling" PCR reaction, there was no hybridisation between the probe and the DNA size markers (Figure 8.6). The gDNA Southern blot gave very promising results, where each lane contained one signal that was stronger than the remainder. The results were so clear that it is probable that further work carried out on this project would be best focussed using this approach.

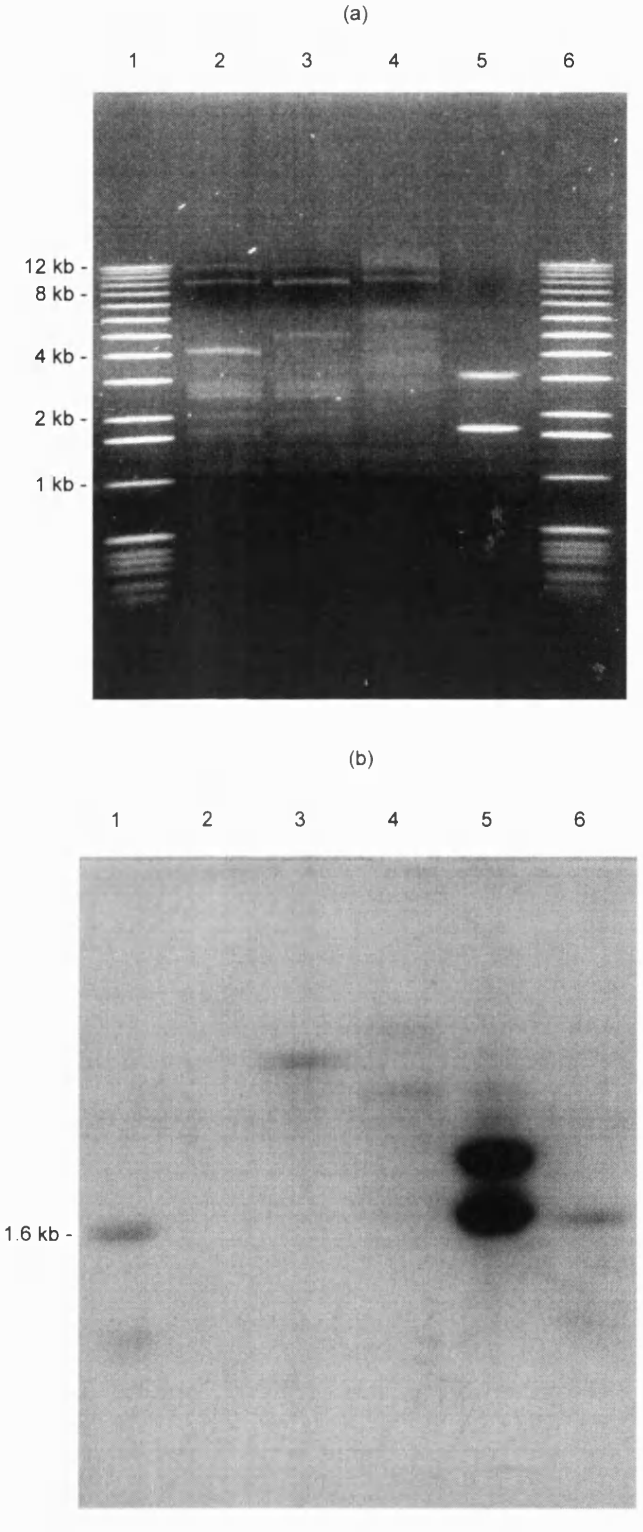


Figure 8.5 Southern blot of *Pst* I digests of λ clones 1 - 3

(a) Agarose gel electrophoresis, lanes 1 and 6, 1 kb ladder, 2 - 4, *Pst* I digests of clones 1 - 3, 5, pGEMT81 clone.

(b) Corresponding autoradiograph after Southern blotting and hybridisation.

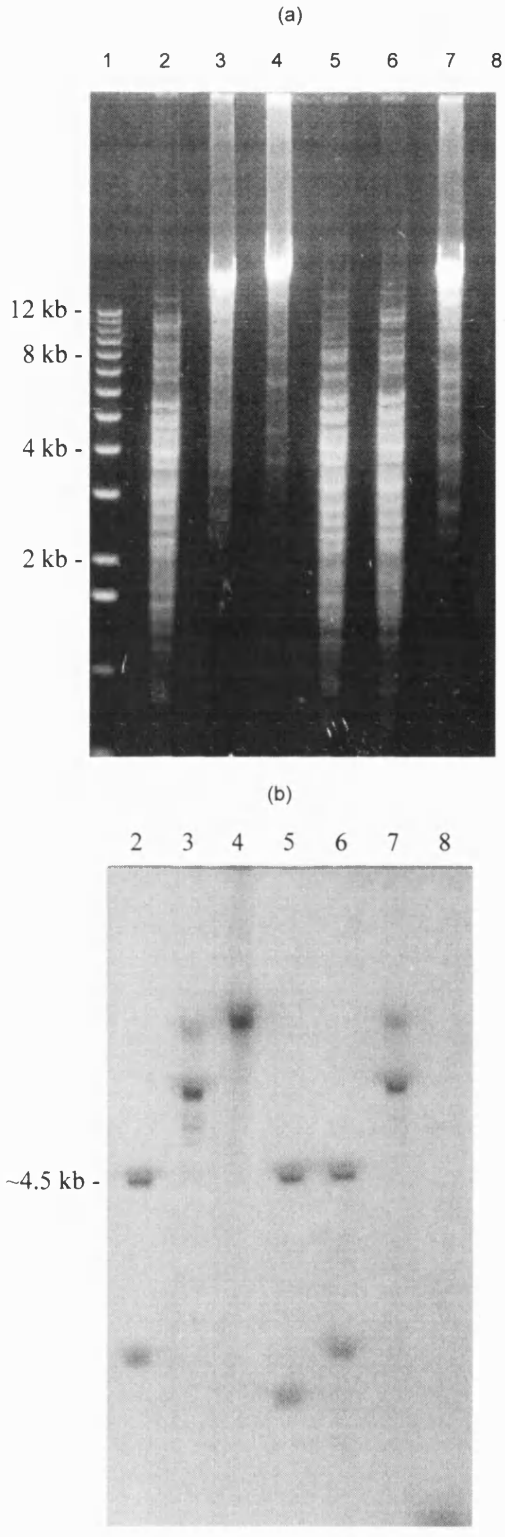


Figure 8.6 Southern blot of single digests of *T. natronophilum* gDNA

(a) Agarose gel electrophoresis, lane 1, 1 kb ladder, 2, gDNA digested with *Eco* RI, 3, *Hind* III, 4, *Xho* I, 5, *Eco* RI/*Hind* III, 6, *Eco* RI/*Xho* I, 7, *Hind* III/*Xho* I and 8, 81 bp PCR product (not visable on gel photograph). (b) Corresponding autoradiograph after Southern blotting and hybridisation.

8.5 DISCUSSION

The failure of the previous labelling procedure, which was carried out using the High prime® method, seemed to be due to the binding of numerous primers to the same template and thus the production of a number of small probes instead of the expected 81 bp probe. The obvious solution to this problem was to use primers already used to produce the 81 bp PCR product in a second PCR reaction, in the presence of a radiolabelled nucleotide.

Due to the numerous products formed in the PCR reaction using the degenerate primers and *T. natronophilum* gDNA as template, it was necessary to use the pGEMT81 clone as template, thus eliminating any possibility of amplification of non-specific probes from gDNA. However, when the radiolabelled probe was used in a screening procedure not only did it hybridise with the 81 bp PCR product (positive control), it also hybridised with the DNA molecular weight markers. This was later discovered to be due to the composition of the markers, which are derived from the 2μ plasmid from yeast, which itself was within a plasmid, pMB9 [Hartley and Donelson 1980, Bolivar *et al.* 1977]. Thus, it appeared that the hybridisation to the markers was due to the presence of the MCS (Multiple Cloning Site), which is also present in the pGEMT plasmid. Therefore, it would seem that in the initial stages of amplification of the 81 bp α-amylase fragment, some of the MCS was being amplified and thus contained the radiolabelled nucleotide, causing the false-positive signal. This problem was eliminated with the introduction of a further step in the production of the gene specific probe. A PCR reaction was carried out using the pGEMT81 clone and the primers CRT03 and CRT08, from which the 81 bp was extracted and purified by separation by electrophoresis through agarose and subsequent extraction using the MERmaid® kit. This purified 81 bp PCR product was then used as template in the labelling PCR reaction. This resulted in the production of a gene specific probe which did not show any hybridisation to the DNA molecular weight markers.

Following the final optimisation of the probe, gDNA southern blots appeared to yield excellent results, from which it would be within the realms of possibility to clone and express the gene responsible for the α-amylase gene from *T. natronophilum*.

8.6 CONCLUSIONS

A suitable labelling procedure has been developed for the isolation of the α-amylase gene using an 81 bp nucleotide fragment obtained using amino acid sequence data. This labelling procedure appears to be ideal for fragments that are unsuitably small for the High prime® kit. The resulting probe shows good specificity when probing gDNA, which is impressive when taking into account the amount of DNA present.

Unfortunately, due to time constraints, it was not possible to continue practical work. Hopefully, this work will be continued at a later date and the α-amylase will be cloned using the information recorded within this report.

General conclusions

- *Thermopallium natronophilum* possesses an α-amylase and a pullulanase type-I.

- The α-amylase is active over a broad temperature range (<65 - 90 °C) at which over 50 % of the optimal activity is observed. The enzyme is also active at high pH (8.5 - 11.5) which makes the hydrolase ideal for the detergent industry.

- The pullulanase has a more neutral pH optimum (pH 7.5) and slighlty higher temperature optimum than the α-amylase which possibly makes it more suitable to the food industry, namely the production of High Fructose Corn Syrup (HFCS).

- A PCR based approach to labelling of small fragments is more efficient compared to the random-primed approach of the HIGH PRIME® labelling kit.

- This work has arrived at a stage where the isolation of the *T. natronophilum* α-amylase gene is a realistic goal.

- The techniques leading to the identification of a gene fragment from a Southern blot using the α-amylase probe could be used to isolate the pullulanase gene from *T. natronophilum.*

Future Work

Having developed a procedure to produce a specific probe from a short nucleotide sequence and identifying a single fragment, which almost certainly contains whole or part of the α-amylase from *Thermopallium natronophilum*. I would obviously like to see the project proceed towards the isolation and determination of the nucleotide sequence α-amylase gene. This could be achieved by performing an *Eco* RI restriction digest of *T. natronophilum* gDNA. Fragments corresponding to 4 - 5 kb could be isolated after separation of gDNA digests by agarose gel electrophoresis and ligated into a suitable dephosphorylated *Eco* RI cut vector (*Eg.* pUC18). Screening of subsequent tranformed competent *E. coli* cells (JM109) would be achieved using the PCR labelling approach using the 81 bp PCR product. Following the identification of the clone containing the α-amylase gene, large plasmid preparations would be obtained to provide an adequate amount of template DNA for subsequent sequencing reactions, starting with the primers designed from the 81 bp PCR sequence.

I would hope that this information would possibly resolve why the α-amylase has low specific activity compared to the pullulanase from *T. natronophilum* and α-amylases from other sources. From a biotechnological point of view, I would like to see the α-amylase gene subcloned (*Eg.* pET system vectors) and over-expressed so that large quantities of the enzyme can be amassed for detergent trials and an assessment of the enzyme's suitability to this industry.

I would also like to see the same procedures developed in this work to be used to isolate the gene responsible for the pullulanase, although the biotechnological prospects for pullulanases in general are not as well founded as they are for amylases.

Once the α-amylase and pullulanase genes have been isolated and characterized, they could be compared with each other on a molecular scale, using sequence alignment software (*Eg.* Pileup, GCG). From a bioengineering approach, the importance of certain residues within the catalytic site that confer substrate specificity, could be investigated.

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Appendix I

Assay Validation

Assay conditions were identical to those in section 2.1.4.1. except 100µl of enzyme sample was incubated with 650 µl of 20 mM potassium phosphate buffer (pH 6.9), 1 % (w/v) soluble potato starch and 2 mM $CaCl_2$ at 70 °C over 20 min. U in this section are defined as 1 mg maltose liberated per 3 min under assay conditions.

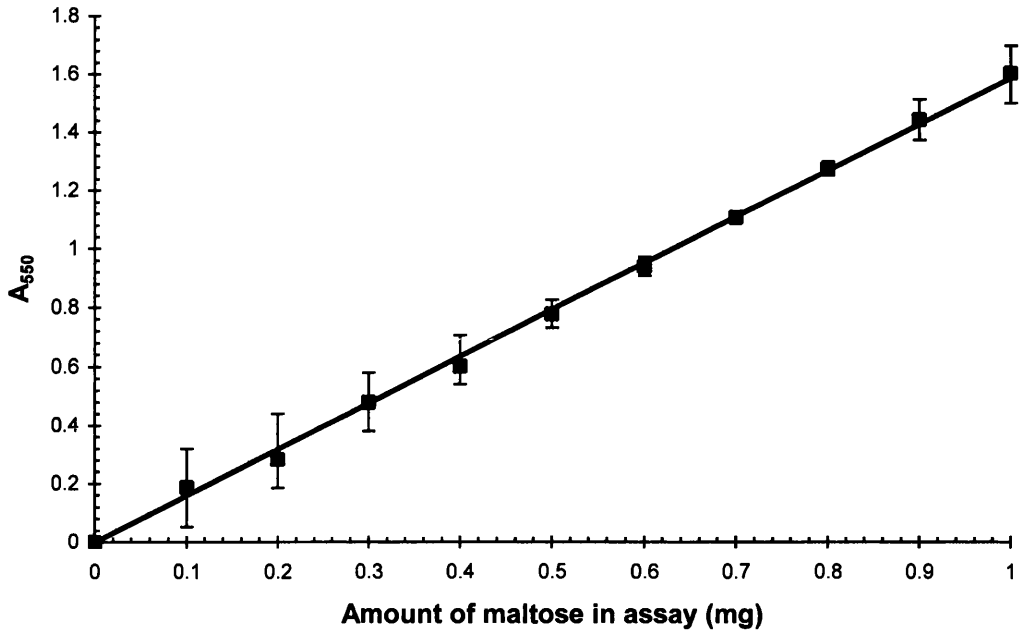

Figure 1 Relataionship between reducing sugar concentration and absorbance at 550 nm.
Error bars represent upper and lower values from 2 complete and one incomplete datasets.

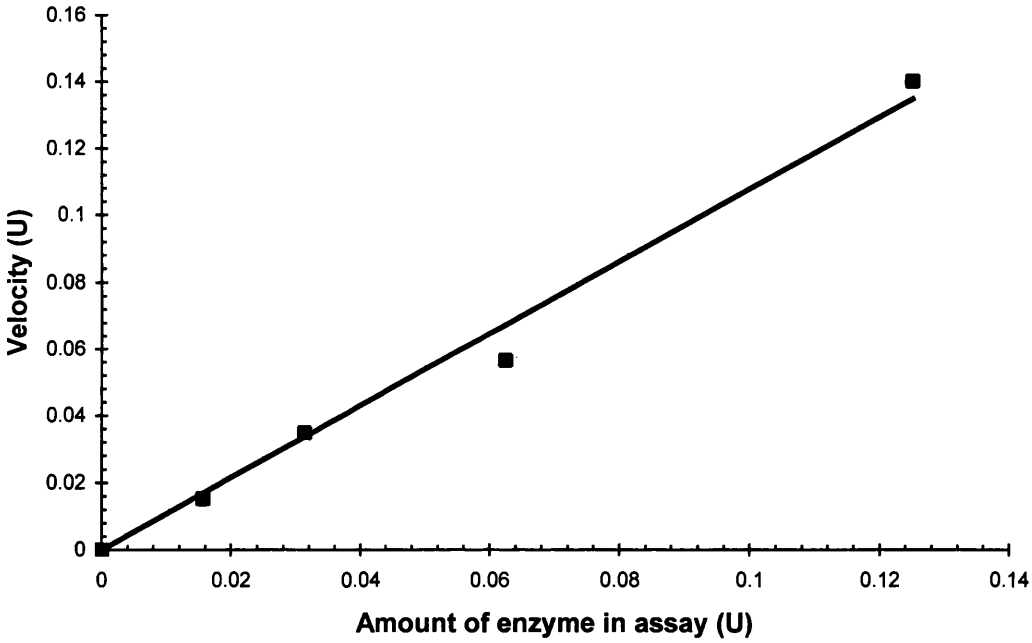

Figure 2 Relationship between the amount of enzyme present in the assay and velocity.

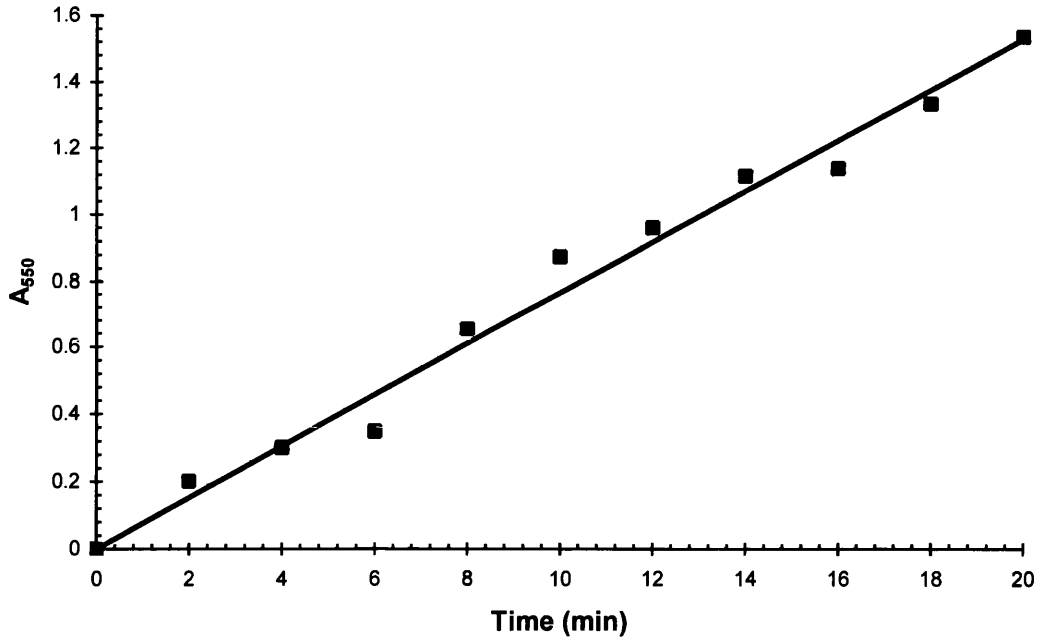

Figure 3 Relationship between assay incubation time and absorbance at 550 nm.

11 assays each containing 0.125 U of α-amylase (*B. licheniformis*) were performed and developed at two minute intervals.

Appendix II

World patent - **"Novel alkaliphilic and thermophilic micro-organisms and enzymes obtained therefrom" (WO 97/10342)**

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/56, 15/31, 1/20, 9/28, 9/44 // (C12N 1/20, C12R 1:01)

A1

(11) International Publication Number: WO 97/10342

(43) International Publication Date: 20 March 1997 (20.03.97)

(21) International Application Number: PCT/EP96/03896

(22) International Filing Date: 3 September 1996 (03.09.96)

(30) Priority Data:
95202477.6 13 September 1995 (13.09.95) EP
(34) Countries for which the regional or international application was filed: NL et al.

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(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.
With an indication in relation to a deposited microorganism furnished under Rule 13bis separately from the description.
Date of receipt by the International Bureau:
21 October 1996 (21.10.96)

(54) Title: ALKALIPHILIC AND THERMOPHILIC MICROORGANISMS AND ENZYMES OBTAINED THEREFROM

(57) Abstract

The present invention provides thermophilic alkaliphilic bacteria and thermostable alkaline polypeptides obtainable therefrom. It also provides a method for producing polypeptides according to the invention, nucleic acids encoding such polypeptides and compositions comprising such polypeptides. It also relates to the use of enzymes obtainable from these novel organisms in the detergent industry, the paper and pulp industry and the textile industry.

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2745P

NOVEL ALKALIPHILIC AND THERMOPHILIC MICROORGANISMS AND ENZYMES OBTAINED THEREFROM

TECHNICAL FIELD OF THE INVENTION

The present invention relates to novel alkaliphilic and thermophilic microorganisms and to novel enzymes obtained therefrom.

BACKGROUND OF THE INVENTION

Alkaliphiles are a heterogeneous group of microorganisms spread over many taxonomic groups which exhibit optimum growth in an alkaline pH environment (Jones, B.E. *et al*, (1994) Alkaliphiles: diversity and identification, in "Microbial Diversity and Identification" (F.G. Priest *et al*, Eds.) Plenum Press, New York and London, pages 195-230), generally in excess of pH 8. Obligate alkaliphiles generally have a pH optimum for growth between pH 9 and pH 10, and are incapable of growth at neutral pH. Alkalitolerant microorganisms are less exacting and although they are capable of growth at alkaline pH values, their optima lie in the neutral to acid pH range.

Thermophiles are also a very heterogeneous collection of microorganisms defined as having an optimum growth temperature in excess of 50°C. For moderate thermophiles the maximum growth temperature usually lies below 70°C. An organism with a growth minimum above 40°C, an optimum above 65°C, and a growth maximum above 70°C is defined as an extreme thermophile (Cowan, D.A. (1992) Biochemistry and molecular biology of extremely thermophilic archeaobacteria, in "Molecular Biology and Biotechnology of Extremophiles" (R.A.

Herbert and R.J. Sharp, Eds.), Blackie & sons Ltd., Glasgow and London, pages 1-43).

The combined phenotype, alkaliphily and thermophily appears to have only rare occurrence. Only two such microorganisms, both isolated from sewage digestion plants, have been well described and both were assigned to the genus *Clostridium* of the Gram-positive bacteria. One of the organisms, *Clostridium paradoxum*, is obligately alkaliphilic growing between pH 7.3 and pH 11.0, with an optimum around pH 10. It can however, only be classified as a moderate thermophile since it has an optimum growth temperature of 55°C and a maximum at 63°C (Youhong Li *et al* (1992) Int. J. Syst. Bacteriol. **43**, 450-460). A second organism, *Clostridium thermoalcaliphilum* is a facultative alkaliphile or alkalitolerant organism growing between pH 7 and pH 11, with an optimum between pH 9.5 and pH 10. With an optimum growth temperature of 50°C and maximum at 57°C this bacterium can only be classified as a very moderate thermophile or as thermotolerant (Youhong Li *et al* (1994) Int. J. Syst. Bacteriol. **44**, 111-118).

Among the known types of thermophilic bacteria several species belong to the order *Thermotogales*. This distinct group of mainly extreme thermophilic bacteria has been shown by sequencing of the ribosomal RNA genes to be phylogenetically distant from all other bacteria, and to represent one of the deepest branches and most slowly evolving lineages within the Domain *Bacteria*. Bacteria of the *Thermotogales* are characteristically, Gram-negative, rod-shaped, anaerobic, fermentative bacteria with an outer sheath-like envelope ("toga"); growth is inhibited by molecular hydrogen (Huber, R. and Stetter, K.O. (1992) The order *Thermotogales*, in "The Prokaryotes" (A. Balows *et al*, Eds.), Springer-Verlag, New York, pages 3809-3815).

At present, the *Thermotogales* are represented by five genera. The genera *Thermotoga, Thermosipho* and *Fervidobacterium* encompass the known extreme thermophilic species, while the more distantly related (on the basis of 16S rRNA analysis) genera *Geotoga* and *Petrotoga* represent the more mesophilic species. None of the known species is noticeably alkaliphilic in nature. Most of the extant

species of extreme thermophilic *Thermotogales* have been isolated from active geothermal aquatic environments such as shallow and deep-sea marine hydrothermal systems or from low-salinity continental solfatara springs. More recently less thermophilic strains, particularly those of the genera *Geotoga* and *Petrotoga* have been isolated from deep sub-surface oil fields (Huber, R. and Stetter, K.O. (1992) ibid; Davey, M.E. *et al*, (1993) Syst. Appl. Microbiol. **16**, 191-200; Ravot, G. *et al*, (1995) Int. J. Syst. Bacteriol. **45**, 308-314).

Although the different members of the *Thermotogales* may be partially differentiated on the basis of phenotypic characters such as temperature, pH and NaCl range permitting growth (Table 1, Ravot, G. *et al* (1995) Int. J. Syst. Bacteriol. **45**, 308-314) the classification is largely based on a comparison of similarity between nucleotide sequences on the 16S rRNA genes and DNA-DNA hybridisation studies. Stackebrandt and Goebel (Int. J. Syst. Bacteriol. **44**, 846-849, 1994) indicate that strains of microorganisms having more than 97% 16S rRNA sequence identity may be considered members of the same species, provided that other criteria are also met. It has been shown that the 16S rRNA sequences of *Fervidobacterium islandicum* and *Fervidobacterium nodosum* are 95.3% similar which is typical of different species within the same genus (Huber, R. *et al*, (1990) Arch. Microbiol. **154**, 105-111), but that these differ by 10-15% with strains of *Thermotoga* and *Thermosipho*. Within the *Thermotogales* sequence differences of up to about 8% have generally qualified for placing the strains in the same genus. 16S rRNA sequence differences of greater than about 10%, together with differences in phenotype have frequently been used as compelling arguments for placing different isolates of *Thermotogales* in separate genera (Huber, R. *et al*, (1989) Syst. Appl. Microbiol. **12**, 32-37; Davey, M.E. *et al*, (1993) Syst. Appl. Microbiol. **16**, 191-200; Ravot, G. *et al*, (1995) Int. J. Syst. Bacteriol. **45**, 308-314).

Table 1. Some Characteristics that Differentiate members of the *Thermotogales*

		TEMPERATURE °C		pH		NaCl CONCENTRATION (%)		G + C	
GENUS	SPECIES	RANGE	OPTIMUM	RANGE	OPTIMUM	RANGE	OPTIMUM	CONTENT (mol%)	REFERENCE
Thermotoga	*maritima*	55-90	80	5.5-9	6.5	0.25-3.75	2.7	46	1
	neapolitana	55-90	80	5.5-9	7			41	2
	thermarum	55-84	70	5.5-9	7	0.2-0.55	0.35	40	3
	elfii	50-72	66	5.5-8.7	7.5	0-2.8	1.2	39.6	4
	sp.FjSS3	55-90	80	4.8-8.2	7			45.8	5
Thermosipho	*africanus*	35-77	75	6-8	7.2	0.11-3.6		29	6
Fervidobacterium	*nodosum*	41-79	70	6-8	7		0.1	33.7	7
	islandicum	50-80	65	6-8	7.2		0.2	41	8
	pennavorens		70		6.5			40	9
Petrotoga	*miotherma*	35-65	55	5.5-9	6.5	0.5-10	2	39.8	10
Geotoga	*petraea*	30-55	50	5.5-9	6.5	0.5-10	3	29.5	10
	subterranea	30-60	45	5.5-9	6.5	0.5-10	4	29.9	10
Thermopallium	*natronophilum*	52-78	70	7.2->10.5	9.2	0-5	1	36.3	11

1. Huber, R. *et al* (1986) Arch. Microbiol. **144**, 324-333.
2. Jannasch, H. *et al* (1988) Arch. Microbiol. **150**, 103-104.
3. Windburger, E. *et al* (1989) Arch. Microbiol. **151**, 506-512.
4. Ravot, G. *et al* (1995) Int. J. Syst. Bacteriol. **45**, 308-314.
5. Huser, B.A. *et al* (1986) FEMS Microbiol. Letts. **37**, 121-127; Janssen, P.H. and Morgan, H.W. (1992) FEMS Microbiol. Letts. **96**, 213-218.
6. Huber, R. *et al* (1989) Syst. Appl. Microbiol. **12**, 32-37.
7. Patel, B.K. *et al* (1985) Arch. Microbiol. **141**, 63-69.
8. Huber, R. *et al* (1990) Arch. Microbiol. **154**, 105-111.
9. WO 93/18134
10. Davey, M.E. *et al* (1993) Syst. Appl. Microbiol. **16**, 191-200.
11. The microorganisms of the present invention.

SUMMARY OF THE INVENTION

The present invention provides novel thermophilic alkaliphilic bacteria of the novel genus *Thermopallium*, more specifically of the novel species *Thermopallium natronophilum*, and novel polypeptides obtainable from these bacteria. In a more specific aspect, the invention provides a novel alkaline amylase preparation from these novel bacteria.

In a third aspect, the invention provides a composition comprising a novel polypeptide according to the invention.

In a fourth aspect, the invention provides an isolated DNA fragment encoding a polypeptide according to the invention, recombinant DNA comprising such DNA fragment, host cells transformed with such recombinant DNA and a culture of such host cells.

In another aspect, the invention provides a method for producing a polypeptide, preferably an enzyme, according to the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the phylogenetic tree based on 16S rDNA sequences illustrating the relationship between the new bacterium *Thermopallium natronophilum* and other bacteria, including members of the order *Thermotogales*.

Figure 2 shows the relation between temperature and enzyme activity (% relative) of the unpurified amylase obtained from *Thermopallium natronophilum* Tg9A, determined after 20 minutes at pH 8.5.

Figure 3 shows the relation between pH and enzyme activity (% relative) of the unpurified amylase obtained from *Thermopallium natronophilum* Tg9A, determined after 20 minutes at 80°C.

Figure 4 shows the relation between temperature and enzyme activity (% relative) of Amylase A-I, determined after 20 minutes at pH 8.5.

Figure 5 shows the relation between pH and enzyme activity (% relative) of Amylase A-I, determined after 20 minutes at 80°C.

Figure 6 shows the relation between temperature and enzyme activity (% relative) of Amylase A-II, determined after 20 minutes at pH 8.5.

Figure 7 shows the relation between pH and enzyme activity (% relative) of Amylase A-II, determined after 20 minutes at 80°C.

DETAILED DISCLOSURE OF THE INVENTION

The microorganisms

The novel microorganisms of the present invention were isolated from hot springs and their run-off streams having an alkaline pH due to dissolved carbonate (and related anions) but having a low concentration of dissolved salts as measured by electrical conductivity (Table 2). The hot springs were located in the volcanically active regions of the Rift Valley in continental East Africa and by having typical carbonate anion concentrations in excess of 1 g/l were not typical of the usual solfatara type. A pure culture of the isolated microorganism designated *Thermopallium natronophilum* Tg9A has been deposited on 21 September 1994 according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures at the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany under Accession Number DSM 9460.

Table 2. Characteristics of hot, alkaline springs containing *Thermopallium*.

SITE	TEMPERATURE °C	pH	CONDUCTIVITY mS/cm	*Thermopallium*
1	66	9.5	8	strain Tg7A1
2	85	10	18	-
3	96	8.5	4.8	strain Tg9A
4	60-80	9.8	35	-
5	60-80	9.8	37	-

The microorganism of this invention is a strictly anaerobic, rod-shaped bacterium which does not form endospores. The bacterial cell is surrounded by a characteristic sheath-like outer structure or "toga" balloning over both poles of the cell. The cells usually occur singly or in short chains of up to 3 cells long. During the growth phase the cells can become curved or irregular in shape, and sometimes form aggregates. During the stationary growth phase the cells become coccoid, with one or more cells inside a large spherical body. The microorganism forms round, shiney, whitish, translucent colonies on alkaline nutrient agar or Gelrite, containing carbonate. On the basis of these characteristics the strains of the microorganism *Thermopallium natronophilum* can therefore be assigned to the bacterial order *"Thermotogales"* (Huber, R. and Stetter, K.O. (1992) The order *"Thermotogales"* in: The Prokaryotes, (A. Barlows et al., eds.), Springer-Verlag, New York, pp. 3809-3815).

The natural isolates of the microorganism of the present invention can be further described by the following characteristics.

Growth temperature: grows between 52° and 78°C, no growth at 50°C or at 79°C. The maximum growth rate was observed at 70°C whereas the maximum cell yield was obtained at 63°-64°C.

Growth pH: pH range: 7.2 - >10.5

pH optimum: 8.8 - 9.5

Influence of NaCl: optimum concentration for growth = 1% (w/v), no growth above 4-5% (w/v).

Gram reaction: negative.

KOH reaction: negative.

Aminopeptidase reaction: negative.

Effect of SDS:
in the presence of sodium dodecylsulphate 1%, (w/v) both cells and the the sheath-like structures disappear under the microscope within a few seconds.

Effect of lysozyme:
when lysozyme (10 mg/ml) was added to a suspension of cells under the microscope, little effect was observed. At 20 mg/ml lysozyme some of the rod-shaped cells became spherical.

Growth on	glucose:	positive.
	galactose:	positive.
	maltose:	positive.
	xylose:	weak.
	ribose:	negative.
	formate:	negative.
	acetate:	positive.
	lactate:	negative.
	propionate:	negative.
	pyruvate:	weak.
	glutamate:	negative.
	glycine:	positive.

glycerol:	positive.
ethanol:	negative.
cellulose:	positive.
casein:	positive.
gelatine:	positive.
xylan:	positive.
starch:	positive.
olive oil:	positive.
tryptone:	positive.

Influence of sulphur and hydrogen on growth:

growth is inhibited by H_2 which may be relieved by the addition of sulphur to the medium.

G+C content: 36.3 ± 0.9 Mol% (n=2) (hplc method).

Classification and Identification of the microorganism

The strains were classified on the basis of phylogenetic relationships by direct sequencing of the 16S rRNA genes amplified by PCR. Sequences were compared with sequences for known bacteria accessed from GenBank and EMBL databases. Sequences were aligned and subjected to phylogenetic analysis using programs in versions 3.4 and 3.5c of the PHYLIP package (Felsenstein, J. (1989) Cladistics **5**, 164-166). Similarity values were computed (Table 3) and a phylogenetic tree constructed (Figure 1).

The results indicate that the strains have a 16S rRNA sequence similarity of 98.7% and thus may be considered as isolates of the same species. The results further indicate that the strains of the new microorganism are most closely related to the genus *Fervidobacterium*. However, the strains of the new microorganism have an outer sheath-like structure, often referred to as the 'toga' which is expanded over both poles of the rod-shaped cells. This 'toga' is a common feature of members of the genera *Thermotoga, Thermosipho, Geotoga* and *Petrotoga*

Balows *et al.*, Eds.) Springer-Verlag, New York, p. 3809-3815; Ravot, G. *et al.*, (1995) International Journal of Systematic Bacteriology **45**, 308-314). In contrast, *Fervidobacterium* species have a terminal 'spheroid' (Huber, R. and Stetter, K.O. *ibid*; Huber, R. *et al.*, (1990) Archives of Microbiology **154**, 105-111; Patel, B.K.C. *et al.*, (1985) Archives of Microbiology **141**, 63-69). This evidence alone indicates that the strains of the new microorganism represent a novel species. However, the difference in sequence homology of almost 10% with *Fervidobacterium* is highly significant since these lineages which represent one of the deepest branches of the domain *Bacteria* (Winkler, S. and Woese, C.R. (1991) Systematic & Applied Microbiology **13**, 161-165) are evolving slowly relative to other bacterial lineages (Huber, R. *et al.*, Systematic & Applied Microbiology **12**, 32-37). This indicates that the new bacterium represents strains of a separate and hitherto unknown genus. On this basis the microorganism is assigned to the new genus, *Thermopallium*; and the strains of the microorganism to the species, *Thermopallium natronophilum*. The genus *Thermopallium* and the species *Thermopallium natronophilum* is defined by the nucleotide sequence of the 16S rRNA gene (SEQ ID No. 1 of the attached sequence listing) and the phenotypic characteristics described herein.

The difference in G+C value compared with those of known species (Table 4) further supports the assignment of these new strains of the microorganism to a new species. The phenotypic characteristics of the microorganisms of the present invention clearly set them apart from the known species of the *Thermotogales* (Table 1). The novel microorganisms are clearly extreme thermophiles and have a temperature profile typical of *Thermotogales* species isolated from continental hot springs (i.e. non-marine sources). However, the known species of the *Thermotogales* all have pH optima for growth of around neutrality. In contrast, the novel microorganisms of this invention are clearly obligately alkaliphilic and are unable to grow at neutral pH (Table 1) or without a medium containing carbonate anions.

Table 3. 16S rDNA similarity values

	1	2	3	4	5	6	7	8	9	10
1. Thermopallium natronophilum Tg9A										
2. Thermopallium natronophilum Tg7A1	98.7									
3. Fervidobacterium islandicum		90.9								
4. Fervidobacterium pennavorens		90.7	98.8							
5. Fervidobacterium nodosum		90.7	96.4	96.1						
6. Thermosipho africanus		88.4	90.3	90.4	89.5					
7. Thermotoga maritima		85.9	87.4	87.8	88.0	91.0				
8. Thermotoga thermarum		84.6	87.1	87.2	86.5	90.6	92.5			
9. Geotoga petraea		83.0	82.4	83.1	83.0	82.5	82.5	81.4		
10. Petrotoga miotherma		80.6	81.3	81.3	81.8	80.8	80.0	78.9	86.6	

Table 4. G+C values (Mol%)

1.	Thermopallium natronophilum	36.3
2.	Fervidobacterium islandicum	40
3.	Fervidobacterium pennavorens	40.0
4.	Fervidobacterium nodosum	33.7
5.	Thermosipho africanus	30
6.	Thermotoga maritima	46
7.	Thermotoga thermarum	40
8.	Geotoga petraea	29.5
9.	Petrotoga miotherma	39.8

Cultivation of the microorganism

The microorganism of the present invention can be cultivated only under strictly anoxic conditions in a Freter type anaerobic cabinet or in closed bottles using the strictly anaerobic techniques described by Balch et al. (Microbiol. Rev. (1979), **43**, 260-296). A suitable nutrient medium is composed of an assimilable carbon and nitrogen source together with other essential nutrients provided that the total dissolved salts concentration does not exceed a conductivity value of about 15 mS/cm, and is prepared under the strictly anaerobic conditions indicated above and with the addition of a reductant to give a sufficiently low initial redox value, the medium being composed according to the principles of the known art.

Since the natural isolates of the novel microorganisms of the novel species *Thermopallium natronophilum* of the present invention are alkaliphilic and are unable to grow below pH 7.2, the cultivation is preferably conducted at alkaline pH values which can be achieved by the addition of suitable buffers such as sodium carbonate, or more preferably mixtures of sodium carbonate and sodium bicarbonate, after sterilization of the growth medium, and preferably under a head space gas phase of O_2-free N_2. Such a medium is TMZ-medium which is a modification of *Thermus* medium using Castenholtz salts (Williams, R.A.D. and Da Costa, M.S. (1992) The genus *Thermus* and related microorganisms, in: The Prokaryotes (A. Barlows et al., eds.), Springer-Verlag, New York, p. 3745) adapted to the original conditions in the hot spring water from which the microorganism was isolated in pure culture. Growth is possible in other buffer mixtures such as TRIS/HCl or Borax/NaOH provided that the pH is adjusted with carbonate. Little or no growth is obtained on media adjusted to alkaline pH values with NaOH.

For cultivation on a large scale it is necessary to sparge the medium continuously using O_2-free nitrogen gas.

After fermentation, liquid enzyme concentrates may be obtained by separation of the cells from the culture broth and concentration of the broth using methods known in the art. Alternatively, the cells may be suspended in a suitable liquid and the cells broken open, or disintegrated, or dissolved, to release enzymes

in a soluble fraction, using appropriate methods. After concentrating the solublised enzymes, they may be purified, and/or precipitated in a solid form by the use of salts or water miscible solvents or removal of water. The purified enzymes may finally be obtained in a crystalline form.

Enzymes from the microorganism

The enzymes of this invention are obtainable by the cultivation of a microorganism of the invention, preferably *Thermopallium natronophilum* Tg9A, DSM 9460, or a variant or mutant thereof, in an appropriate nutrient medium poised at an alkaline pH, pH 7.5 to 12.0, or more preferably pH 8.5 to 9, by the addition of carbonate, or mixtures of carbonate and bicarbonate, containing carbon and nitrogen sources and inorganic salts.

The enzymes may also be obtained by recombinant DNA technology by cloning the appropriate genes in a suitable host organism. This may be achieved by, e.g., digesting chromosomal DNA with one or more restriction enzymes to create a genomic library or random DNA fragments of one size. The DNA fragments from the library of the random fragments may be inserted into a vector, which can be a plasmid, a bacteriophage or any other construct which is suitable for the transfer, or for the transfer and expression, of nucleic acid sequences.

Vectors may replicate either after integration into the host cell genome or extrachromosomal, such a in the case of plasmids. Both prokaryotic and eukaryotic microbes, and plants may be used as a host cell.

Recombinant clones may be selected for by using generally available techniques, such as screening for the presence of a marker. Other suitable screening methods include nucleic acid hybridisation, antibody assays or plate assays for the detection of protein activity.

If the enzyme of interest is secreted, it may be recovered from the growth medium by conventional techniques. Alternatively, it can be recovered from the host cells by disrupting them.

Thermopallium natronophilum, the novel microorganisms of this invention has been found to produce valuable novel enzymes, in particular, amylases,

cellulases, lipases, proteases, pullulanases and xylanases. As an illustration, the properties of the amylases of the invention will be described.

Properties of the amylases produced by the microorganism

The amylases produced by *Thermopallium natronophilum* have the following properties.

The molecular weight of the soluble amylase fraction separated from the growth medium by gel-filtration chromatography is 90 kDa, by comparison with protein markers.

This amylase fraction has an optimum temperature for amylase activity of 95°C and an optimum pH of 8.8 (Figure 2 and Figure 3).

Two amylases are separated by ion exchange chromatography and give the following molecular weights when examined by SDS-polyacrylamide electrophoresis:

Amylase A-I subunit molecular weight = 87 kDa

Amylase A-II subunit molecular weight = 83 kDa

Amylase A-I has an optimum temperature of 95°C and optimum pH of 9.6 (Figure 4 and Figure 5). The half-life of activity is 11 minutes measured at 96°C.

Amylase A-II has an optimum temperature of 80°C and optimum pH of 10.2 (Figure 6 and Figure 7). No enzyme activity is lost by incubation at 80°C for 120 minutes.

The amylases are further characterised:

Amylase A-I :
- Hydrolytic activity on soluble starch producing primarily maltose and other dextrins.
- Hydrolytic activity on pullulanan.
- Activity is enhanced by NaCl (0.0067M)

Amylase A-II :
- Hydrolytic activity on soluble starch producing dextrins

(G1-G9).

- Activity reduced by EDTA and EGTA.
- Requires Ca^{2+} for activity.

Amylase A-I has a N-terminal amino acid sequence:
Xaa-Xaa-Glu-Ile-Ile-Tyr-Val/Asp-Gly-Phe

and contains the (internal) partial amino acid sequence:
Tyr-Ile-Gly-Asp-Gly-Ala-(Trp)-Glu-Ala-Val-Leu-Glu-Gly-(Asp)-(Asp)-Glu-(Gly/Glu)-Phe-Tyr-Arg.

Amylase A-II comprises the (internal) partial amino acid sequence:
Ile-Gly-Leu-Pro-Ser-Val-Met-Thr-Glu-Pro-Trp-Asn-Pro-Ile-Gly-Gly-Ser-Asn-(Trp)-Ile-Phe-Asp-Met-Met-Leu-Ile-(Arg).

Utility of the invention

Enzymes obtained from the novel organisms may be used in the detergent industry in laundry detergents and automatic dishwashing detergents. Due to their thermostability and alkaline nature, these enzymes are extremely suitable to be used at high temperatures at high pH. In other words, they are extremely suitable to be used under conditions which are ideal for washing, especially for dishwashing. Examples of enzymes which may be used in both powder and liquid detergents for the degradation of stains and soil are amylases for the degradation of carbohydrates, proteases for the degradation of protein and lipases for the degradation of lipids.

The detergent industry is not the only industry interested in thermostable alkaline enzymes. Many useful applications for these enzymes are also found in the paper and pulp industry and the textile industry. For instance, thermostable alkaline amylases may be used for laundry detergents and automatic dishwashing detergents, but also for the manufacture of paper, especially desizing, and disizing of textiles, especially in combination with an alkaline scouring process.

It is this versatility of thermostable alkaline enzymes that explains the growing demand for these enzymes.

EXAMPLES

Example 1

Cultivation of the microorganism

Medium

Thermopallium natronophilum was cultivated in a medium having the following composition (per liter):

100 ml	Solution A	
10 ml	Solution B	
10 ml	Solution C	
5 ml	Vitamin solution	(Raven et al. (1992) Appl. Microbiol. Biotech. **38**, 263-267. or DSM 141).
1 ml	Resazurin solution	(1 g/L) (Sigma)
2 g	Tryptone (Difco Bacto)	
1 g	Yeast extract (Difco Bacto)	
2.5 g	Starch, soluble (BDH/Merck)	
2 g	Sodium chloride	
5 g	Sodium bicarbonate	
0.5 g	Sodium sulphide.xH_2O	

The pH was adjusted to pH 8.5 with 1M HCl or 20% v/v H_2SO_4.
The medium was prepared under strictly anaerobic conditions.

Large scale growth conditions

Cultures were grown in 20 liter volumes in glass reservoir bottles without pH control, at 65°C, under continuous sparging with oxgen-free nitrogen (0.1 vvm) through a P160 glass distribution tube (maximum porosity 160 μm). The sterile medium was inoculated with 1% of a pre-grown culture.

Cells were grown for 18-21 hours, achieving an optical density (A_{600}) of 0.7-0.8. The cells were harvested by serial centrifugation (5000 rpm, 20 min., 4°C) in a Sorvall RC3-B centrifuge. A typical biomass yield was 3.2-3.9 g/L wet weight. The cell paste was stored at -20°C.

Example 2

Enzyme extraction

The cell paste (from Example 1) was diluted to 0.2 g/ml in buffer (0.05 M Tris, 0.005 M EDTA), pH 8.5). The mixture was sonicated at 0°C using a 3 mm probe in a Ultrasonics Ltd., model SP-958 apparatus by applying 3 x 10 seconds of 50 W. The broken cell suspension was centrifuged at 20,000 rpm for 20 min. at 5°C (Sorvall, rotor SM-24). The cell pellet was re-suspended in buffer, mixed and re-centrifuged. The 2 supernatant fractions were combined.

Alternatively

A concentrated suspension of cells was defrosted and 6 N NaOH was added to raise the pH to 12. The mixture was incubated overnight at room temperature. The pH was re-adjusted with acid to pH 8-10 and the mixture centrifuged at 20,000 rpm for 40 min. and the supernatant collected.

Example 3

Purification of amylase enzymes

The supernatant (from Example 2) was concentrated to 1.5-2 ml by ultra-filtration in a Centriprep-30 unit (Amicon) a membrane having a 10 kDa molecular weight cut-off. The concentrated protein was subjected to gel-filtration on a HR 16/60 Superdex-200 column which was eluted with 0.02 M Tris buffer (pH 8.5) at a flow rate of 1 ml/min. Fractions containing amylase activity were combined and subjected to ion-exchange chromatography on a HR 5/5 Mono Q column. Protein was eluted with a salt gradient of 0-2 M NaCl in 0.02 M Tris buffer, pH 8.5 at a flow rate of 0.75 ml/min. Two fractions of separate amylase activity were obtained

which were combined and dialysed against 0.02 M Tris buffer, pH 8.5. A further purification of the individual amylase proteins was accomplished by a further application of ion-exchange chromatography. Two fractions of amylase activity were obtained, amylase A-I and amylase A-II. The two amylase components were examined by SDS-PAGE which indicated subunit molecular weights of 87 kDa and 83 kDa for A-I and A-II, respectively.

Example 4

Assays for amylase activity

Method 1

A modified Bernfeld assay was used (Bernfeld, P. (1955) in: Methods in Enzymology, vol. 1 (S.P. Colowick and N.O. Kaplan, Eds.) Academic Press, New York, pp 149-158). 100 μl of enzyme sample was incubated at 80°C with 425 μl of buffer (0.05 M Tris, 0.005 M EDTA, 0.0067 M NaCl [pH 8.5 at 20°C, pH 8 at 80°C]), 150 μl of substrate (0.05 M Tris, 0.005 M EDTA, 0.0067 M NaCl, 1% ($^{w}/_{v}$) soluble potato starch (Sigma), pH 8.5 at 20°C) and 75 μl of 0.002 M $CaCl_2$ for 20 minutes. The reaction was terminated with developing solution (1% $^{w}/_{v}$ 3,5-dinitrosalicylic acid in 0.4 M NaOH) and boiled for 5 minutes. The assay mixture was cooled on ice and the absorbance measured at 550 nm and read against a calibration curve constructed using maltose instead of enzyme.

Method 2

100 μl enzyme sample was incubated at 70°C with 900 μl substrate solution (1 % $^{w}/_{v}$ soluble starch in 0.005 M MES/HEPES/glycine buffer, pH 8.0) for 60 minutes. The reaction was terminated by the addition of 10 μl 6 N HCl and developed by the addition of 1 ml of iodine solution (Sigma, P700-2; diluted 1:3) to 100 μl of reaction mixture. The absorbance was measured at 620 nm against water and compared to a standard calibration curve constructed using a standard α-amylase (Maxamyl S3) with an α-amylase activity of 82650 TAU/g.

Method 3 (for amylase A-I)

Conditions were identical to Method 1 except that the enzyme sample was incubated with 650 μl of substrate (0.05 M Tris, 1% $^{w}/_{v}$ soluble potato starch, 0.0067 M NaCl, pH 8.5 at 20°C).

Method 4 (for amylase A-II)

Conditions were identical to Method 1 except that the enzyme sample was incubated with 650 μl substrate solution (0.05 M Tris, 1% $^{w}/_{v}$ soluble potato starch, 0.002 M $CaCl_2$, pH 8.5 at 20°C).

Example 5

Effect of temperature on enzyme activity

In a first test, the unpurified enzyme derived from the grown cells according to Example 2, and separated by gel-filtration chromatography (Example 3) was assayed for amylase activity in the range 60° to 105°C. Enzyme activity was measured in 0.05 M Tris buffer according to assay Method 1 of Example 4.

The results show an optimum temperature for activity of 95°C, as presented in Figure 2.

In a second test, the purified amylase A-I obtained according to Example 3 was assayed in the range 65° to 100°C. Enzyme activity was measured according to assay Method 3 of Example 4.

The results show an optimum temperature for activity of 95°C, with 50% of the maximum activity displayed in the range 88° to 99°C, as shown in Figure 4.

In a third test, the purified amylase A-II obtained according to Example 3 was assayed in the range 65° to 100°C. Enzyme activity was measured according to assay Method 4 of Example 4.

The results show a broad profile with an optimum temperature for activity of 80°C, with 50% of the maximum activity in the range <65° to 96°C, as shown in Figure 6.

Example 6

Effect of pH on enzyme activity

In a first test, the unpurified enzyme derived from the grown cells according to Example 2, and separated by gel-filtration chromatography (Example 3) was assayed for amylase activity in a pH range from pH 6.0 to 10.8. Enzyme activity was measured at 80°C according to assay Method 1 of Example 4, and by substituting Tris buffer with an appropriate buffer (MES, HEPES or glycine) for the pH range required.

The results show a broad optimum between pH 7.5 and pH 9.5 with a maximum activity of pH 8.8, as presented in Figure 3.

In a second test, the purified amylase A-I obtained according to Example 3 was assayed in the pH range 4.1 to 11.4 by employing appropriate buffers (acetate, MOPS, MES, Tris, HEPES, diethanolamine or glycine). Enzyme activity was measured at 80°C according to assay Method 3 of Example 4.

The results show an optimum pH for activity of amylase A-I of pH 10.2, as presented in Figure 5.

In a third test, the purified amylase A-II obtained according to Example 3 was assayed in the pH range 4.1 to 11.4 by employing appropriate buffers (acetate, MOPS, MES, Tris, HEPES, diethanolamine or glycine). Enzyme activity was measured at 80°C according to assay Method 3 of Example 4.

The results show an optimum pH for activity of amylase A-II of pH 9.6, with 50% of the maximum activity in the range pH8.1 to >pH11.5, as presented in Figure 7.

Example 7

Amino acid sequence analysis of the amylases

The N-terminal amino acid sequence of the amylase A-I having a molecular weight of 87 kDa, obtained according to Example 3 was determined by EUROSEQUENCE (Groningen, The Netherlands).

The N-terminal amino acid sequence (SEQ ID No. 1 of the attached sequence listing) was assigned as:

Xaa-Xaa-Glu-Ile-Ile-Tyr-Val/Asp-Gly-Phe.

A further fragment of the amylase A-I protein yielded an amino acid sequence (SEQ ID No. 2 of the attached sequence listing) as follows:

Tyr-Ile-Gly-Asp-Gly-Ala-(Trp)-Glu-Ala-Val-Leu-Glu-Gly-(Asp)-(Asp)-Glu-(Glu/Gly)-Phe-Tyr-Arg.

Similarly, amylase A-II, a protein of 83 kDa, obtained according to Example 3 provided a partial amino acid sequence (SEQ ID No. 3 of the attached sequence listing) as follows:

Ile-Gly-Leu-Pro-Ser-Val-Met-Thr-Glu-Pro-Trp-Asn-Pro-Ile-Gly-Gly-Ser-Asn-(Trp)-Ile-Phe-Asp-Met-Met-Leu-Ile-(Arg).

Example 8

Isolation of amylase genes

A genomic library of the strain Tg9a was constructed in plasmid pTZ18R (Mead, D.A. et al. (1986) Protein Engineering **1**, 67). Chromosomal DNA of *Thermopallium natronophilum* Tg9A was digested with *Hind*III and *Eco*R1. Restriction fragments were size fractionated by agarose gel electrophoresis and fragments of 1 kb and greater were isolated from the gel. This fraction was ligated to *Hind*III/*Eco*R1 digested DNA from the vector pTZ18R. The ligate was transformed to *E. coli* XL1 Blue MRF by electroporation. Recombinant clones were screened on amylose azure agar. Clones that exhibited clearing zones around the colony were isolated. The amylase activity of the recombinant strains was determined after growth for 24 hours at 37°C in LB-medium (Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, page 433). The plasmid DNA of the recombinant strains was isolated and the inserts characterised by restriction analysis.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Gist-brocades B.V.
(B) STREET: Wateringseweg 1
(C) CITY: Delft
(E) COUNTRY: The Netherlands
(F) POSTAL CODE (ZIP): 2311 XT

(ii) TITLE OF INVENTION: Novel Alkaliphilic and Thermophilic Microorganisms and Enzymes Obtained Therefrom

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1437 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Thermopallium natronophilum

(B) STRAIN: Tg9A

(C) INDIVIDUAL ISOLATE: DSM 9460

(ix) FEATURE:

(A) NAME/KEY: misc_RNA

(B) LOCATION: 1..1437

(D) OTHER INFORMATION: /partial

/product= "16S ribosomal RNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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AGCATAGCGG CGGACGGGTG AGTAATACAC AGGAACGTGC CCCTTGGAGG CGGATAGCTG 120

TGGGAAACTG CAGGTAATCC GCCGTAAGCT CGGGAGAGGA AAGCCGGAAG GCGCCGAGGG 180

AGCGGCCTGT GGCCCATCAG GTAGTTGGTA GGGTAAGAGC CTACCAAGCC GACGACGGGT 240

AGCCGGTCTG AGAGGATGGA CGGCCACAAG GGCACTGAGA CACGGGCCCT ACTCCTACGG 300

GAGGCAGCAG TGGGGGATAT TGGACAATGG GCGAAAGCCT GATCCAGCGA CGCCGCGTGA 360

GGGACGAAGT CCTTCGGGAC GTAAACCTCT GTTGTAGGGG AAGAAGACAG TGACGGTACC 420

CTACGAGGAA GCCCCGGCTA ACTACGTGCC AGCAGCCGCG GTAATACGTA GGGGNCGAGC 480

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GACCACGGCT CAACCGTGGG GATGGGCTGG AAACTGTTAG ACTTGAGGGC ACTAGAGGCA 600

GACGGAACTG CTGGTGTAGG GGTGAAATCC GTAGATATCA GCAGGAACGC CGGTGGAGAA 660

GTCGGTCTGC TGGGGTGACC CTGACGCTGA GGCACGAAAG CTAGGGGAGC GAACCGGATT 720

AGATACCCGG GTAGTCCTAG CCGTAAACGA TGCTCACTAG GTGTGGGGGA GTAAATCCTC 780

CGTGCTGAAG CTAACGCGAT AAGTGAGCCN CCTGGGGAGT ACGCCCNCAA GGGTGAAACT 840

CAAAGGAATT GACGGGGGNC CGCACAANCG GTGGAGCGTG TGGTTTAATT GGAAGCTAAG 900

CCAAGAACCT TACCAGGGTT TGACATTCTG GTGGTACCGA NCCGAAAGGT GAGGGACTCT 960

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GTGGGCACTC TAAGGGGACT GCCTGCGACG AGCAGGAGGA AGGAGGGGAT GACGTCAGAT 1140

ACTCGTGCCC CTTATGCCCT GGGCGACACA CGCGCTACAA TGGGCAGGAC AAAGGGAAGC 1200

GAGCCGGCGA CGGTGAGCAA ATCCCAAAAA CCTGCCCCCA GTTCAGATTG TGGGCTGAAA 1260

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GTNCCCGGGN CTTGTACACA CCGCCCGTCA AGCCACCCGA GTTGGGGGNA CCCGAAGATA 1380

CGTACCCTTA GGGGGCGTAT TTAGGGTGAA CCTGGTGAGG GGGGGTNGTC GGAAGTC 1437

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Thermopallium natronophilum

(B) STRAIN: Tg9A

(C) INDIVIDUAL ISOLATE: DSM 9460

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Xaa Xaa Glu Ile Ile Tyr Xaa Gly Phe
1 5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Thermopallium natronophilum

(B) STRAIN: Tg9A

(C) INDIVIDUAL ISOLATE: DSM 9460

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Tyr Ile Gly Asp Gly Ala Trp Glu Ala Val Leu Glu Gly Asp Asp Glu
1 5 10 15

Xaa Phe Tyr Arg
20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Thermopallium natronophilum

(B) STRAIN: Tg9A

(C) INDIVIDUAL ISOLATE: DSM 9460

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ile Gly Leu Pro Ser Val Met Thr Glu Pro Trp Asn Pro Ile Gly Gly
1 5 10 15

Ser Asn Trp Ile Phe Asp Met Met Leu Ile Arg
20 25

CLAIMS

1. A pure culture of thermophilic alkaliphilic bacteria wherein the bacteria are members of the strain *Thermopallium natronophilum*, preferably *Thermopallium natronophilum* Tg9A, as deposited under number DSM 9460.

2. A pure culture of thermophilic bacteria, wherein the nucleotide sequence of the 16S rRNA of said bacteria shows 92-100% identity to the 16S rRNA gene sequence as listed in SEQ ID no. 1.

3. A pure culture of thermophilic bacteria according to claim 2, wherein the nucleotide sequence of the 16S rRNA of said bacteria shows 95-100% identity to the 16S rRNA gene sequence as listed in SEQ ID no. 1.

4. A polypeptide from thermophilic bacteria according to any one of claims 1-3 which is obtainable by culturing bacteria capable of producing said polypeptide under conditions conducive of production thereof and recovering said polypeptide.

5. A polypeptide according to claim 4, wherein the polypeptide is an enzyme, preferably an enzyme which exhibits amylase activity.

6. An alkaline amylase preparation having the N-terminal amino acid sequence as listed in SEQ. ID. no. 2 or 4, or the internal sequence as listed in SEQ. ID. 3, or a variant wherein said variant differs by a deletion, substitution or insertion of one or more amino acids, with the proviso that said deletion, substitution or insertion does not abolish the activity of said amylase preparation.

7. A composition comprising a polypeptide according to claims 4-5 or an enzyme preparation according to claim 6.

8. An isolated DNA fragment encoding a polypeptide according to claims 4-5 or an enzyme having an amino acid sequence as listed in SEQ. ID. 2, 3 or 4.

9. Recombinant DNA comprising a DNA fragment according to claim 8.

10. A host cell transformed with recombinant DNA according to claim 9.

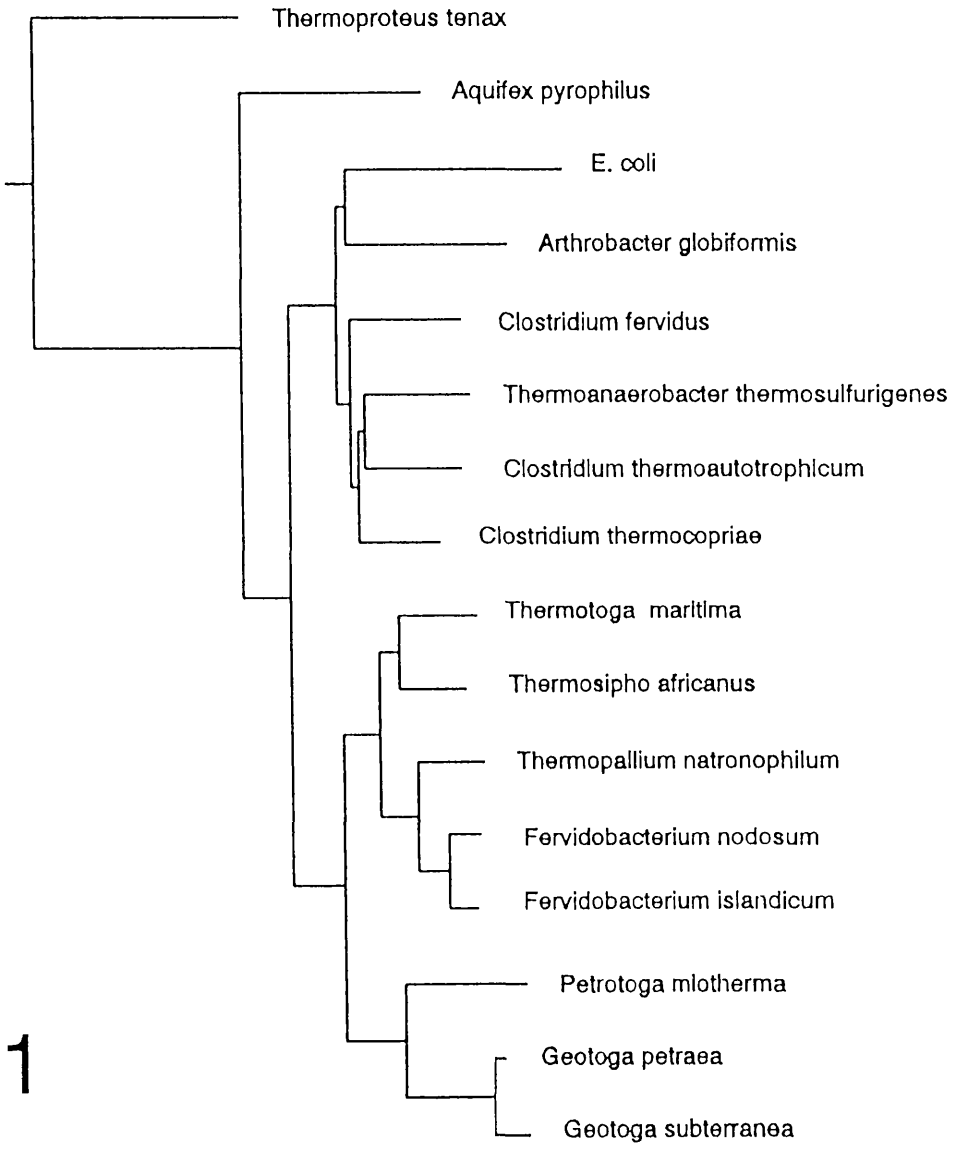
11. A method for producing a polypeptide according to claims 4-5 or an enzyme preparation according to claim 6 comprising culturing cells according to any one of claims 1-3 and recovering the resulting polypeptide.

12. The use of a polypeptide according to claims 4-5 or an enzyme preparation according to claim 6 in the paper and pulp industry, in the textile industry or for the formulation of a detergent composition.

13. A fermentation process using a culture of bacteria according to any one of claims 1-3.

ABSTRACT

The present invention discloses novel thermophilic alkaliphilic bacteria and novel thermostable alkaline polypeptides obtainable therefrom. It also provides a method for producing a polypeptide according to the invention, DNA encoding such polypeptide and a composition containing such polypeptide. It also relates to the use of enzymes obtainable from these novel organisms in the detergent industry, the paper and pulp industry and the textile industry.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Clostridium thermocopriae
Thermotoga maritima
Thermosipho africanus
Thermopallium natronophilum
Fervidobacterium nodosum
Fervidobacterium islandicum
Petrotoga mlotherma
Geotoga petraea
Geotoga subterranea

Figure 1

Figure 2. Effect of Temperature on the Unpurified Amylase Activity

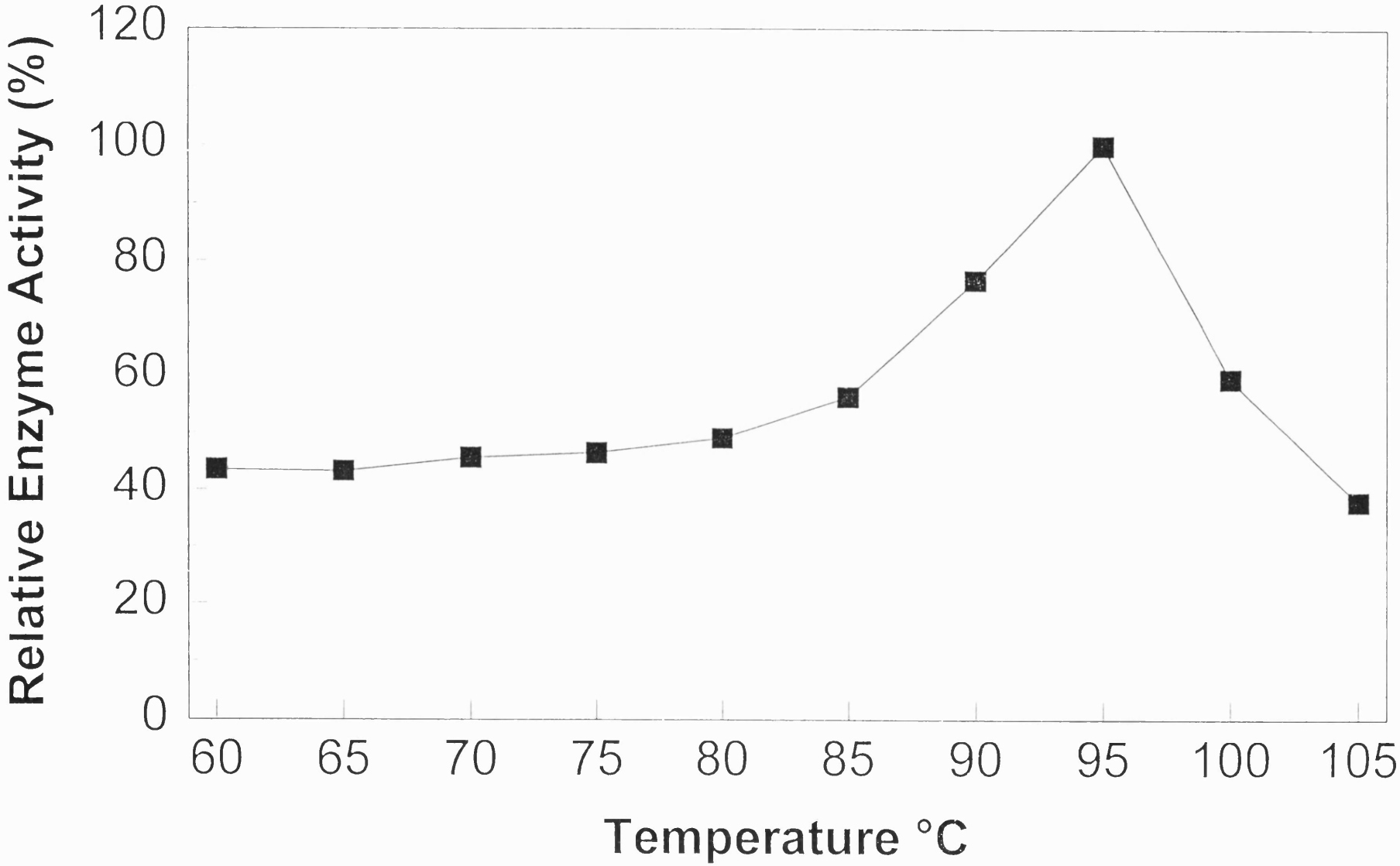

Figure 3. Effect of pH on the Unpurified Amylase Activity

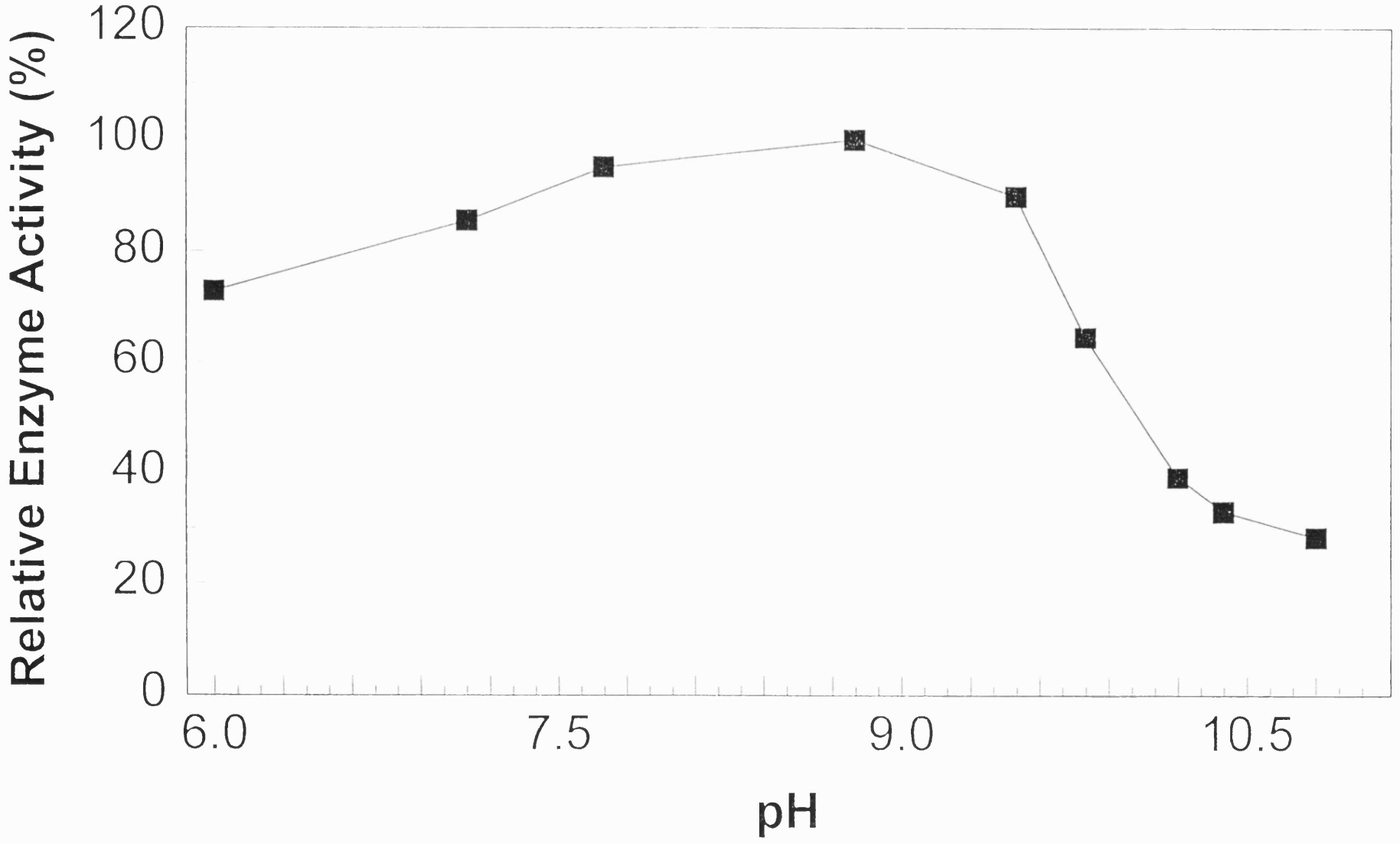

Figure 4. Effect of Temperature on Amylase A-I Activity

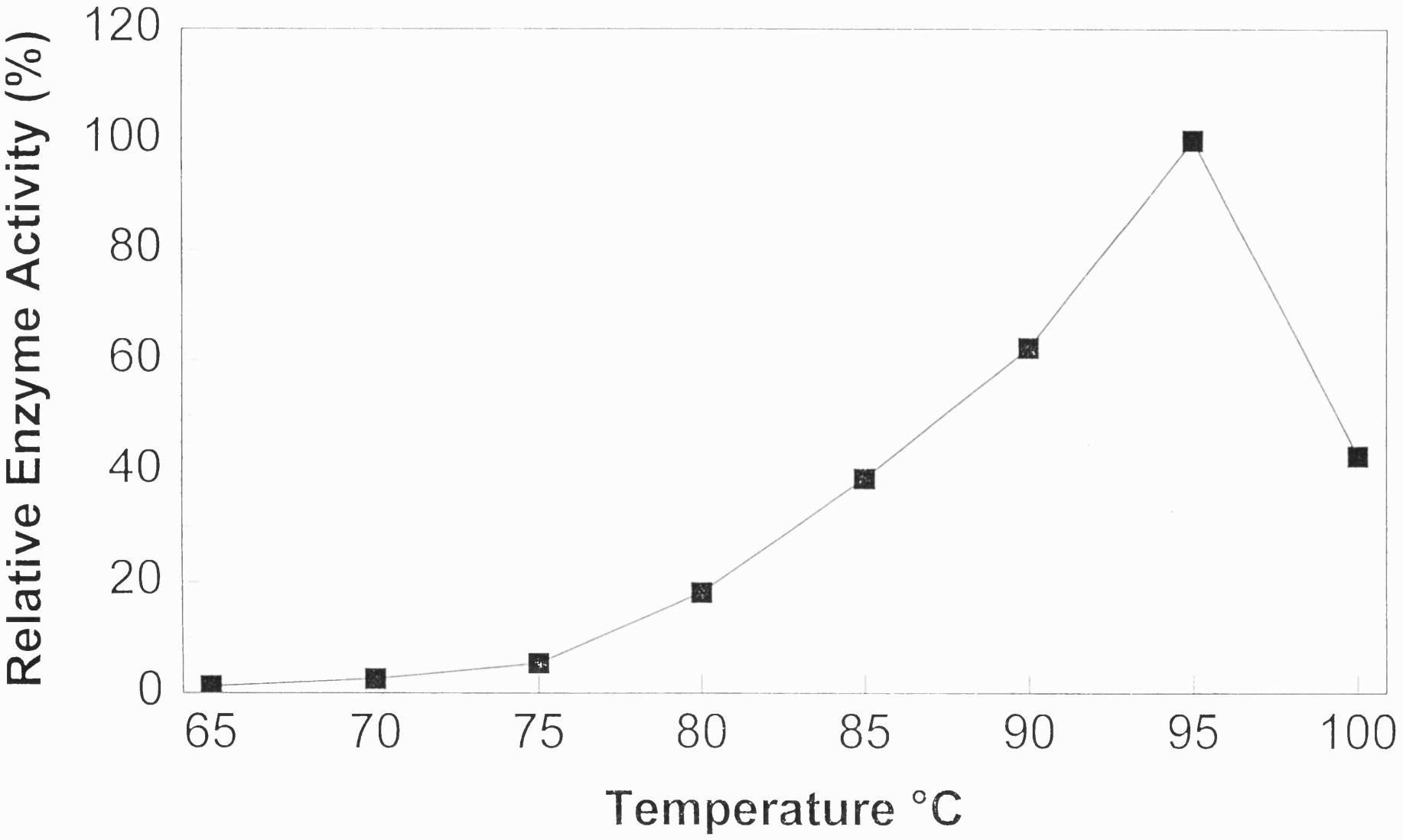

Figure 5. Effect of pH on Amylase A-I Activity

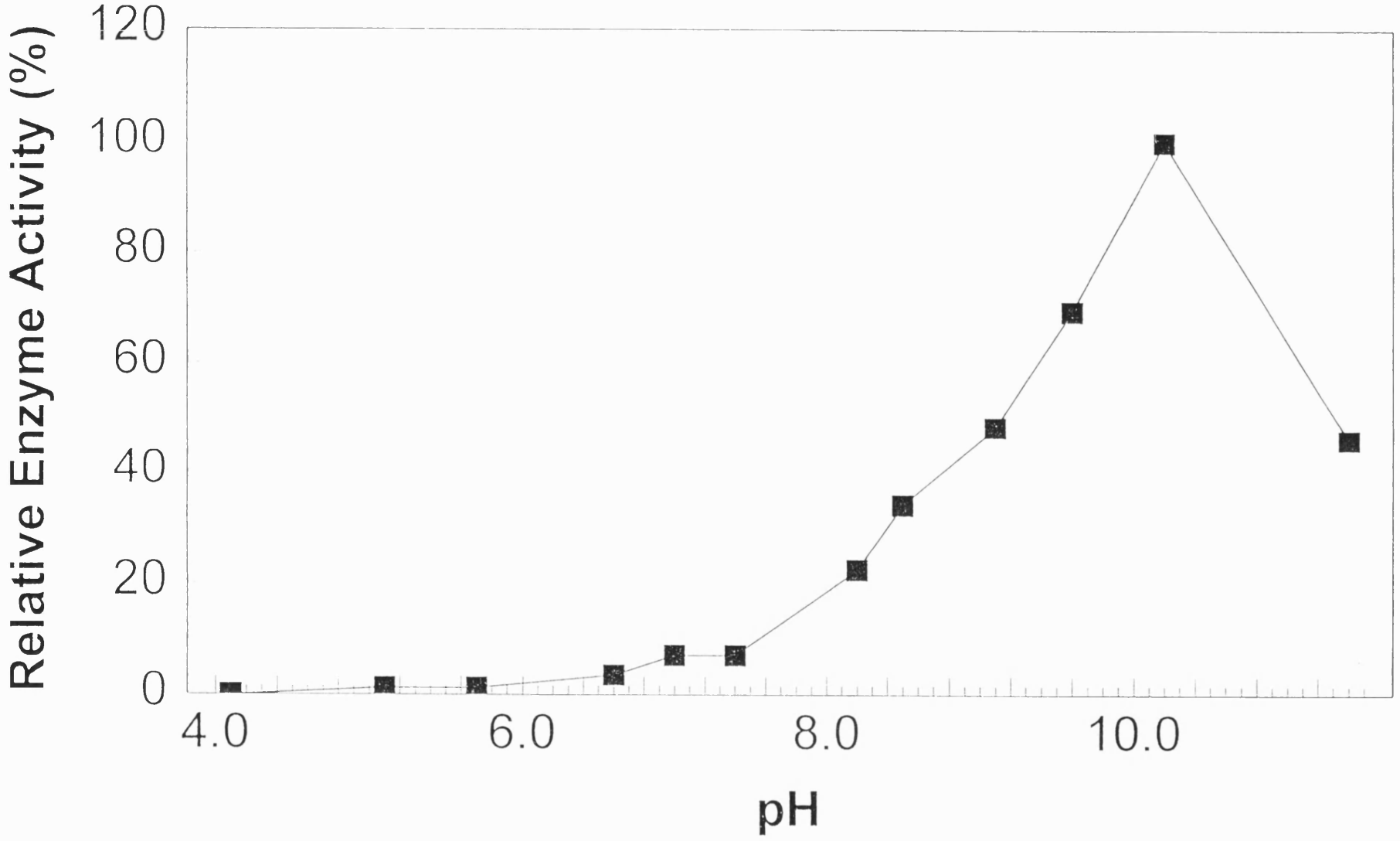

Figure 6. Effect of Temperature on Amylase A-II Activity

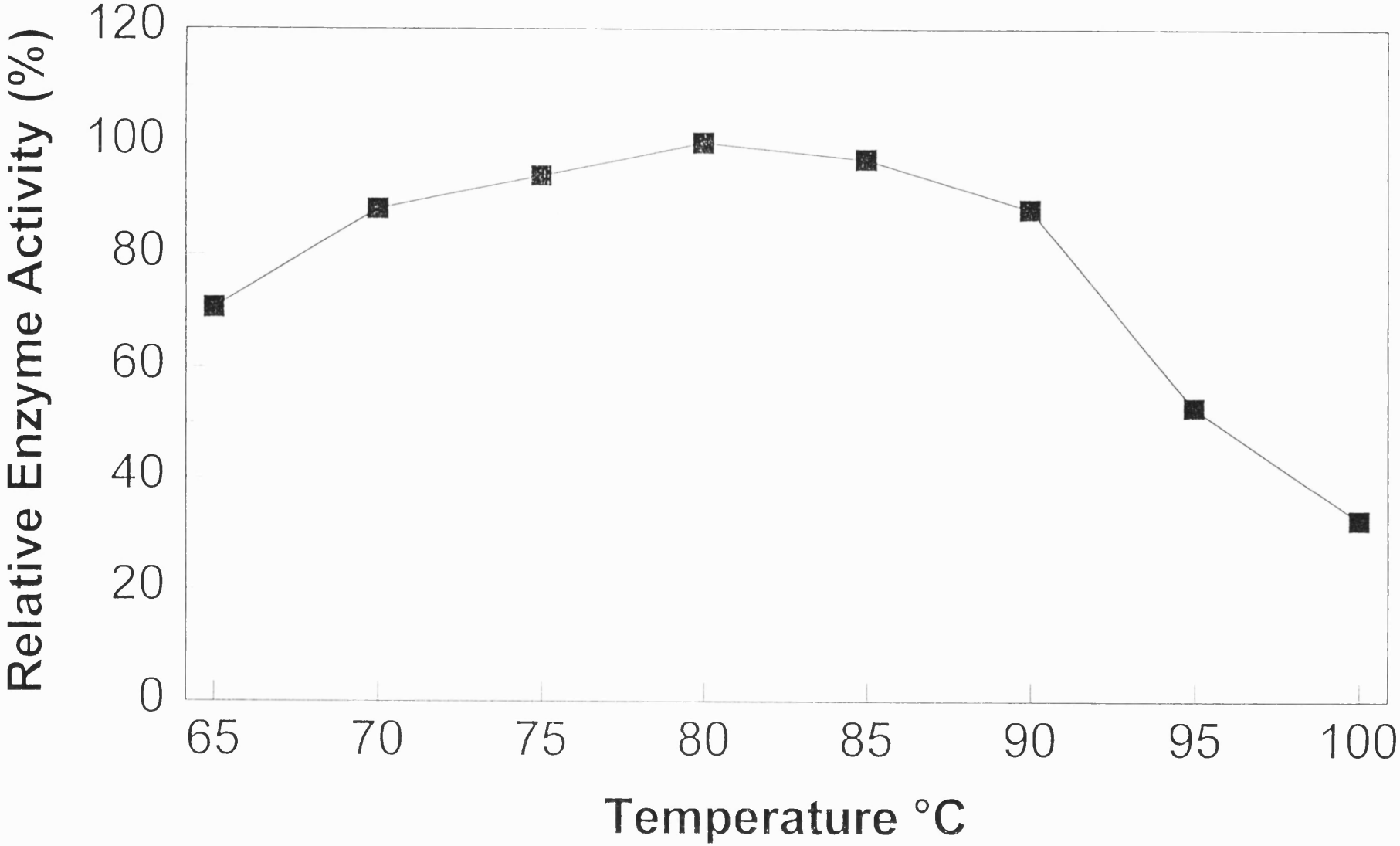

Figure 7. Effect of pH on Amylase A-II Activity